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(54) Title: THERAPY FOR α-GALACTOSIDASE A DEFICIENCY

(57) Abstract

A therapeutic method whereby an individual suspected of having an α-galactosidase A deficiency, such as Fabry disease, is treated either with (1) human cells that have been genetically modified to overexpress and secrete human α-gal A, or (2) purified human α-gal A obtained from cultured, genetically modified human cells.

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THERAPY FOR α-GALACTOSIDASE A DEFICIENCY
Background of the Invention

This invention relates to α-galactosidase A and
5 treatment for α-galactosidase A deficiency.

Fabry disease is an X-linked inherited lysosomal
storage disease characterized by symptoms such as severe
renal impairment, angiokeratomas, and cardiovascular
abnormalities, including ventricular enlargement and
10 mitral valve insufficiency. The disease also affects the
peripheral nervous system, causing episodes of agonizing,
burning pain in the extremities. Fabry disease is caused
by a deficiency in the enzyme α-galactosidase A (α-gal
A), which results in a blockage of the catabolism of
15 neutral glycosphingolipids, and accumulation of the
enzyme's substrate, ceramide trihexoside, within cells
and in the bloodstream.

Due to the X-linked inheritance pattern of the
disease, essentially all Fabry disease patients are male.
20 Although a few severely affected female heterozygotes
have been observed, female heterozygotes are generally
either asymptomatic or have relatively mild symptoms
largely limited to a characteristic opacity of the
cornea. An atypical variant of Fabry disease, exhibiting
25 low residual α-gal A activity and either very mild
symptoms or apparently no other symptoms characteristic
of Fabry disease, correlates with left ventricular
hypertrophy and cardiac disease (Nakano et al., New Engl.
J. Med. 333:288-293, 1995). It has been speculated that
30 reduction in α-gal A may be the cause of such cardiac
abnormalities.

The cDNA and gene encoding human α-gal A have been
isolated and sequenced (Bishop et al., Proc. Natl. Acad.
Sci. USA 83:4859, 1986; Kornreich et al., Nuc. Acids Res.
35 17:3301, 1988; Oeltjen et al., Mammalian Genome 6:335-

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338, 1995). Human α-gal A is expressed as a 429-amino
acid polypeptide, of which the N-terminal 31 amino acids
constitute a signal peptide. The human enzyme has been
expressed in Chinese Hamster Ovary (CHO) cells (Desnick,
5   U.S. Patent No. 5,356,804; Ioannou et al., J. Cell Biol.
119:1137, 1992); insect cells (Calhoun et al., U.S.
Patent No. 5,179,023); and COS cells (Tsuji et al., Eur.
J. Biochem. 165:275, 1987). Pilot trials of α-gal A
replacement therapies have been reported, using protein
10 derived from human tissues (Mapes et al., Science 169:987
1970; Brady et al., N. Engl. J. Med. 289:9, 1973; Desnick
et al., Proc. Natl. Acad. Sci. USA 76:5326, 1979), but
there is currently no effective treatment for Fabry
disease.

15   Summary of the Invention
         It has been found that expressing a DNA encoding
human α-gal A in cultured human cells produces a
polypeptide that is glycosylated appropriately, so that
it is not only enzymatically active and capable of acting
20 on the glycosphingolipid substrate which accumulates in
Fabry disease, but is also efficiently internalized by
cells via cell surface receptors which target it exactly
to where it is needed in this disease: the lysosomal
compartment of affected cells, particularly the
25 endothelial cells lining the patient's blood vessels.
This discovery, which is discussed in more detail below,
means that an individual suspected of having an α-gal A
deficiency such as Fabry disease can be treated either
with (1) human cells that have been genetically modified
30 to overexpress and secrete human α-gal A, or (2) purified
human α-gal A obtained from cultured, genetically
modified human cells.
         Therapy via the first route, i.e., with the
modified cells themselves, involves genetic manipulation

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of human cells (e.g., primary cells, secondary cells, or
immortalized cells) *in vitro* or *ex vivo* to induce them to
express and secrete high levels of human α-gal A,
followed by implantation of the cells into the patient,

5 as generally described in Selden et al., WO 93/09222
(herein incorporated by reference).

When cells are to be genetically modified for the
purposes of treatment of Fabry disease by either gene
therapy or enzyme replacement therapy, a DNA molecule

10 that contains an α-gal A cDNA or genomic DNA sequence may
be contained within an expression construct and
introduced into primary or secondary human cells (e.g.,
fibroblasts, epithelial cells including mammary and
intestinal epithelial cells, endothelial cells, formed

15 elements of the blood including lymphocytes and bone
marrow cells, glial cells, hepatocytes, keratinocytes,
muscle cells, neural cells, or the precursors of these
cell types) by standard methods of transfection
including, but not limited to, liposome-, polybrene-, or

20 DEAE dextran-mediated transfection, electroporation,
calcium phosphate precipitation, microinjection, or
velocity driven microprojectiles ("biolistics").
Alternatively, one could use a system that delivers DNA
by viral vector. Viruses known to be useful for gene

25 transfer include adenoviruses, adeno associated virus,
herpes virus, mumps virus, poliovirus, retroviruses,
Sindbis virus, and vaccinia virus such as canary pox
virus. Although primary or secondary cell cultures are
preferred for the therapy methods of the invention, one

30 can also use immortalized human cells. Examples of
immortalized human cell lines useful in the present
methods include, but are not limited to, Bowes Melanoma
cells (ATCC Accession No. CRL 9607), Daudi cells
(ATCC Accession No. CCL 213), HeLa cells and derivatives

35 of HeLa cells (ATCC Accession Nos. CCL 2, CCL 2.1, and

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CCL 2.2), HL-60 cells (ATCC Accession No. CCL 240),
HT1080 cells (ATCC Accession No. CCL 121), Jurkat cells
(ATCC Accession No. TIB 152), KB carcinoma cells
(ATCC Accession No. CCL 17), K-562 leukemia cells
5 (ATCC Accession No. CCL 243), MCF-7 breast cancer cells
(ATCC Accession No. BTH 22), MOLT-4 cells (ATCC Accession
No. 1582), Namalwa cells (ATCC Accession No. CRL 1432),
Raji cells (ATCC Accession No. CCL 86), RPMI 8226 cells
(ATCC Accession No. CCL 155), U-937 cells (ATCC Accession
10 No. CRL 1593), WI-38VA13 subline 2R4 cells (ATCC
Accession No. CLL 75.1), and 2780AD ovarian carcinoma
cells (Van der Blick et al., Cancer Res. 48:5927-5932,
1988) as well as heterohybridoma cells produced by fusion
of human cells and cells of another species. Secondary
15 human fibroblast strains, such as WI-38 (ATCC Accession
No. CCL 75) and MRC-5 (ATCC Accession No. CCL 171), may
also be used.

       Following the genetic engineering of human cells
with a DNA molecule encoding α-gal A (or following
20 another appropriate genetic modification, as described
below) to produce a cell which overexpresses and secretes
α-gal A, a clonal cell strain consisting essentially of a
plurality of genetically identical cultured primary human
cells, or, where the cells are immortalized, a clonal
25 cell line consisting essentially of a plurality of
genetically identical immortalized human cells, may be
generated. Preferably, the cells of the clonal cell
strain or clonal cell line are fibroblasts.

       The genetically modified cells can then be
30 prepared and introduced into the patient by appropriate
methods, e.g. as described in Selden et al., WO 93/09222.

       Gene therapy in accordance with the invention
possesses a number of advantages over enzyme replacement
therapy with enzyme derived from human or animal tissues.
35 For example, the method of the invention does not depend

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upon the possibly inconsistent availability of sources of
appropriate tissues, and so is a commercially viable
means of treating α-gal A deficiency. It is relatively
risk-free compared to enzyme-replacement therapy with
5 enzyme derived from human tissues, which may be infected
with known or unknown viruses and other infective agents.
Furthermore, gene therapy in accordance with the
invention possesses a number of advantages over enzyme
replacement therapy in general. For example, the method
10 of the invention (1) provides the benefits of a long-term
treatment strategy that eliminates the need for daily
injections; (2) eliminates the extreme fluctuations in
serum and tissue concentrations of the therapeutic
protein, which typically accompany conventional
15 pharmacologic delivery; and (3) is likely to be less
expensive than enzyme replacement therapy because
production and purification of the protein for frequent
administration are unnecessary.

        As described above, individuals with α-gal A
20 deficiencies may also be treated with purified α-gal A
(i.e. enzyme replacement therapy). Primary, secondary,
or immortalized human cells genetically modified to
overexpress human α-gal A will also be useful for *in
vitro* protein production, to produce protein which may be
25 purified for enzyme replacement therapy. Secondary or
immortalized human cells may be chosen from among those
described above and may be genetically modified by the
transfection or transduction methods also described
above. After genetic modification, the cells are
30 cultured under conditions permitting overexpression and
secretion of α-gal A. The protein is isolated from the
cultured cells by collecting the medium in which the
cells are grown, and/or lysing the cells to release their
contents, and then applying standard protein purification
35 techniques. One such technique involves passing the

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culture medium, or any sample containing human α-gal A,
over a hydrophobic interaction resin such as Butyl
Sepharose® or another resin having a functional moiety
that includes a butyl group. Passing the sample over
5   such a resin may constitute the first chromatography
step. If further purification is required, the α-gal A-
containing material eluted from the hydrophobic
interaction resin may be passed over a column containing
a second resin, such as an immobilized heparin resin such
10 as Heparin Sepharose®, hydroxyapatite, an anion exchange
resin such as Q Sepharose®, or a size exclusion resin
such as Superdex® 200. Preferably, the purification
protocol would include use of each of the above types of
resins. Alternatively, one could use one or more of the
15 latter resins before or instead of the hydrophobic
interaction resin.

Previous methods for the preparation of α-gal A
with relatively high purity were dependent on the use of
affinity chromatography, using a combination of lectin
20 affinity chromatography (concanavalin A (Con A)
Sepharose) and affinity chromatography based on binding
of α-gal A to the substrate analog N-6-aminohexanoyl-α-D-
galactosylamine coupled to a Sepharose matrix (Bishop et
al., J. Biol. Chem. 256:1307-1316, 1981). The use of
25 proteinaceous lectin affinity resins and substrate analog
resins is typically associated with the continuous
leaching of the affinity agent from the solid support
(cf. Marikar et al., Anal. Biochem. 201:306-310, 1992),
resulting in contamination of the purified product with
30 the affinity agent either free in solution or bound to
eluted protein. Such contaminants make the product
unsuitable for use in pharmaceutical preparations. Bound
substrate analogs and lectins can also have substantial
negative effects on the enzymatic, functional, and
35 structural properties of proteins. Furthermore, such

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affinity resins are typically expensive to prepare,
making the use of such resins less suitable for
production on a commercial scale than more conventional
chromatography resins. Thus, the development of a
5 purification protocol using conventional chromatography
resins, which are readily available in supplies and
quality suitable for large-scale commercial use, is a
significant advantage of the present invention.

An individual who is suspected of having an α-
10 gal A deficiency may be treated by administration of
pharmaceutically acceptable, purified human α-gal A by
any standard method, including but not limited to
intravenous, subcutaneous, or intramuscular injection, or
as a solid implant. The purified protein may be
15 formulated in a therapeutic composition consisting of an
aqueous solution containing a physiologically acceptable
excipient, e.g. a carrier such as human serum albumin, at
pH 6.5 or below.

The present invention thus provides a means for
20 obtaining large quantities of appropriately glycosylated
and therefore therapeutically useful human α-gal A. This
makes enzyme replacement therapy for α-gal deficiency
commercially viable, as well as relatively risk-free
compared to therapy with enzyme derived from human or
25 animal tissues.

Skilled artisans will recognize that the human
α-gal A DNA sequence (either cDNA or genomic DNA), or
sequences that differ from it due to either silent codon
changes or to codon changes that produce conservative
30 amino acid substitutions, can be used to genetically
modify cultured human cells so that they will overexpress
and secrete the enzyme. It is also possible that certain
mutations in the α-gal A DNA sequence will encode
polypeptides that retain or exhibit improved α-gal A
35 enzymatic activity (as would be apparent by expressing

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the mutant DNA molecule in cultured cells, purifying the
encoded polypeptide, and measuring the catalytic
activity, as described herein). For example, one would
expect conservative amino acid substitutions to have

5 little or no effect on the biological activity,
particularly if they represent less than 10% of the total
number of residues in the protein. Conservative
substitutions typically include substitutions within the
following groups: glycine, alanine; valine, isoleucine,

10 leucine; aspartic acid, glutamic acid; asparagine,
glutamine; serine, threonine; lysine, arginine; and
phenylalanine, tyrosine.

        Production of α-gal A by the cells can be
maximized by certain genetic manipulations. For example,

15 the DNA molecule that encodes α-gal A may also encode an
heterologous signal peptide, such as the signal peptide
of human growth hormone (hGH), erythropoietin,
Factor VIII, Factor IX, glucagon, the low density
lipoprotein (LDL) receptor, or a lysosomal enzyme other

20 than α-gal A. Preferably, the signal peptide is the hGH
signal peptide (SEQ ID NO:21), and is at the N-terminus
of the encoded protein. The DNA sequence encoding the
signal peptide may contain an intron such as the
first intron of the hGH gene, resulting in a DNA sequence

25 such as SEQ ID NO:27 (see also Fig. 10). Furthermore,
the DNA molecule may also contain a 3' untranslated
sequence (UTS) that is at least 6 nucleotides in length
(in contrast to the α-gal A mRNA found in humans, which
has no 3' UTS, the requisite polyadenylation site being

30 within the coding sequence). The UTS is positioned
immediately 3' to the termination codon of the coding
sequence, and includes a polyadenylation site. It is
preferably at least 6 nucleotides in length, more
preferably at least 12, and most preferably at least 30,

35 and in all cases it contains the sequence AATAAA or a

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related sequence which serves to promote polyadenylation.
A DNA molecule as described, i.e., encoding an hGH signal
peptide linked to α-gal A and containing a 3' UTS that
includes a polyadenylation site, and preferably including
5 expression control sequences, is also within the
invention. Also within the scope of the invention is a
DNA molecule encoding a protein that includes the signal
peptide of hGH linked to α-gal A or any other
heterologous polypeptide (i.e., any polypeptide other
10 than hGH or an analog of hGH). The heterologous
polypeptide is typically a mammalian protein, e.g. any
medically desirable human polypeptide.

       Other features and advantages of the invention
will be apparent from the detailed description that
15 follows, and from the claims.

       The term "genetically modified," as used herein in
reference to cells, is meant to encompass cells that
express a particular gene product following introduction
of a DNA molecule encoding the gene product and/or
20 regulatory elements that control expression of a coding
sequence. The introduction of the DNA molecule may be
accomplished by gene targeting (i.e., introduction of a
DNA molecule to a particular genomic site); furthermore
homologous recombination allows replacement of the
25 defective gene itself (the defective α-gal A gene or a
portion of it could be replaced in a Fabry disease
patient's own cells with the whole gene or a portion
thereof)).

       The term "α-gal A," as used herein, means α-gal A
30 without a signal peptide, i.e., SEQ ID NO:26 (Fig. 9).
There is some indication that residues 371 to 398 or 373
to 398 of SEQ ID NO:26 (Fig. 9) may be removed in the
lysosome; however, removal of this putative propeptide is
not believed to affect activity of the enzyme. This
35 suggests that any portion of the putative propeptide

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could be deleted without affecting activity. Thus, the
term "α-gal A" as used herein also covers a protein
having a sequence corresponding to SEQ ID NO:26 except
lacking up to 28 residues at the C-terminus of that
5 sequence.

By "α-gal A deficiency" is meant any deficiency in
the amount or activity of this enzyme in a patient. The
deficiency may induce severe symptoms as typically
observed in males who are suffering from Fabry disease,
10 or may be only partial and induce relatively mild
symptoms as can be seen in heterozygous female carriers
of the defective gene.

As used herein, the term "primary cell" includes
cells present in a suspension of cells isolated from a
15 vertebrate tissue source (prior to their being plated,
i.e., attached to a tissue culture substrate such as a
dish or flask), cells present in an explant derived from
tissue, both of the previous types of cells plated for
the first time, and cell suspensions derived from these
20 plated cells.

"Secondary cells" refers to cells at all
subsequent steps in culturing. That is, the first time a
plated primary cell is removed from the culture substrate
and replated (passaged), it is referred to as a secondary
25 cell, as are all cells in subsequent passages.

A "cell strain" consists of secondary cells which
have been passaged one or more times; exhibit a finite
number of mean population doublings in culture; exhibit
the properties of contact-inhibited, anchorage dependent
30 growth (except for cells propagated in suspension
culture); and are not immortalized.

By "immortalized cell" is meant a cell from an
established cell line that exhibits an apparently
unlimited lifespan in culture.

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By "signal peptide" is meant a peptide sequence
that directs a newly synthesized polypeptide to which it
is attached to the endoplasmic reticulum for further
post-translational processing and/or distribution.

5   The term "heterologous signal peptide," as used
herein in the context of α-gal A, means a signal peptide
that is not the human α-gal A signal peptide (i.e., that
encoded by nucleotides 36-128 of SEQ ID NO:18). It
typically is the signal peptide of some mammalian protein
10 other than α-gal A.

The term "first chromatography step" refers to the
first application of a sample to a chromatography column
(all steps associated with the preparation of the sample
are excluded).

15   Brief Description of the Drawings

Fig. 1 is a representation of the 210 bp probe
that was used to isolate α-gal A from a human fibroblast
cDNA library (SEQ ID NO:19). The sequence is from exon 7
of the α-gal A gene. The probe was isolated from human
20 genomic DNA by the polymerase chain reaction (PCR). The
regions underlined in the figure correspond to the
sequences of the amplification primers.

Fig. 2 is a representation of the sequence of the
DNA fragment that completes the 5' end of the α-gal A
25 cDNA clone (SEQ ID NO:20). This fragment was amplified
from human genomic DNA by PCR. The regions underlined
correspond to the sequences of the amplification primers.
The positions of the NcoI and SacII restriction
endonuclease sites, which were used for subcloning as
30 described in Example IA, are also shown.

Fig. 3 is a representation of the sequence of
α-gal A cDNA, including the sequence that encodes the
signal peptide (SEQ ID NO:18).

Fig. 4 is a schematic map of pXAG-16, an α-gal A
35 expression construct that includes the

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CMV (cytomegalovirus) promoter and intron, the hGH signal
peptide coding sequence and first intron, the cDNA for α-
gal A (i.e., lacking the α-gal A signal peptide sequence)
and the hGH 3' UTS.

5   Fig. 5 is a schematic map of pXAG-28, an α-gal A
expression construct that includes the collagen Iα2
promoter, a β-actin intron, the hGH signal peptide coding
sequence and first intron, the cDNA for α-gal A (i.e.,
lacking the α-gal A signal peptide sequence) and the hGH
10 3' UTS.

        Fig. 6 is a chromatogram of the α-gal A
purification step using Butyl Sepharose® resin. The
absorbance at 280 nm (plain line) and α-gal A activity
(dotted line) of selected fractions is shown.

15   Fig. 7 is a line graph depicting internalization
by Fabry fibroblasts of human α-gal A prepared in
accordance with the invention. The intracellular α-gal A
activity and total protein concentration were measured
after incubation of the cells with increasing
20 concentrations of human α-gal A prepared in accordance
with the invention. The effects of the potential
internalization-inhibitors mannose-6 phosphate (M6P; open
diamonds) and mannan (open circles) are shown.

        Fig. 8 is a schematic diagram of the experimental
25 paradigm designed to examine Fabry fibroblasts following
internalization of α-gal A. The α-gal A activity of the
Fabry cells is measured after exposure to either normal
or α-gal A-overexpressing human fibroblasts cultured in
Transwell™ inserts. "M6P" = mannose-6 phosphate;
30 "Untrf HF" = untransfected human fibroblasts; "BRS11" = a
transfected, α-gal A-overexpressing fibroblast strain.

        Fig. 9 is a representation of the human α-gal A
amino acid sequence (SEQ ID NO:26).

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        Fig. 10 is a representation of the DNA sequence
encoding the hGH signal peptide and containing the first
hGH intron (underlined) (SEQ ID NO:27).
        Fig. 11 is a representation of the DNA sequence
5   encoding the hGH signal peptide without the intron (SEQ
ID NO:22).
        Fig. 12 is a representation of the amino acid
sequence of the hGH signal peptide (SEQ ID NO:21).
        Fig. 13 is a representation of the cDNA sequence
10   encoding human α-gal A (without signal peptide)
(SEQ ID NO:25).

                    Detailed Description
        Lysosomal enzymes such as α-gal A are targeted to
the lysosomal compartment of a cell through interaction
15   with the mannose-6-phosphate (M6P) receptor, which binds
to M6P residues present in the oligosaccharide moieties
of enzymes destined for the lysosomal compartment
(Kornfeld, S. and Mellman, I, Ann. Rev. Cell Biol. 5:483-
525, 1989). The primary interaction occurs in the Golgi,
20   where enzymes bound to Golgi M6P receptors are segregated
for transport to the lysosomes. A secondary type of
interaction is believed to take place between
extracellular α-gal A and M6P receptors at the cell
surface. Extracellular substances internalized by cells
25   are transported through the cytoplasm in endocytic
vesicles, which fuse with primary lysosomes and empty
their contents into the lysosomes. In this process, cell
surface M6P receptors are also incorporated into
endocytic vesicles and transported to lysosomes.
30   · Any α-gal A present in the extracellular milieu
can, if it bears M6P residues, bind to the cell surface
M6P receptors and thereby be transported into the
lysosomal compartment along with the receptors. Once in
the lysosomal compartment as a result of this scavenger

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pathway, the enzyme can carry out its appropriate
function. Thus, even if a cell is genetically deficient
in producing its own α-gal A, there exists a mechanism
for it to take up exogenously produced enzyme, provided
5 that (a) the enzyme is suitably glycosylated and (b) the
deficient cell bears M6P receptors.

        In Fabry disease, vascular endothelial cells of
the kidney and heart have been shown to display severe
histopathologic abnormalities and contribute to the
10 clinical pathology of the disease; these cells, which do
carry M6P receptors, are a particular target of the
presently claimed invention. The α-gal A produced in
accordance with the invention may be delivered either
locally or systemically to the affected cells, by gene
15 therapy (i.e., by genetically modified cells which
express and secrete the glycosylated enzyme within the
patient), or by conventional pharmacologic routes of
administration. An α-gal A in which M6P is present in
the N-linked oligosaccharides is therefore of great
20 importance for therapy in accordance with the invention.

        Furthermore, the degree to which the N-linked
oligosaccharides of α-gal A are modified by sialylation
is also of great importance. In the absence of
appropriate sialylation, α-gal A will be rapidly cleared
25 from the circulation due to binding by hepatic
asialoglycoprotein receptors, followed by internalization
and degradation by hepatocytes (Ashwell and Harford, Ann.
Rev. Biochem. 51:531-554, 1982). This decreases the
amount of α-gal A available in the circulation for
30 binding to M6P receptors on cells which contribute to the
clinical pathology of Fabry disease, such as the vascular
endothelial cells of the kidney and heart. Surprisingly,
the Applicants have found that α-gal A secreted by stably
transfected human cells has glycosylation properties
35 which are suitable for the treatment of Fabry disease by

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either gene therapy or by conventional pharmaceutical
administration of the purified secreted protein. This is
in contrast to the situation with the best-studied
lysosomal enzyme, glucocerebrosidase, in which delivery
5 of the enzyme purified from human placenta or secreted
from transfected CHO cells to the clinically-relevant
cells in the body requires complex enzymatic modification
of the enzyme following purification (cf. Beutler, New
Engl. J. Med. 325:1354-1360, 1991).
10   The therapy of the invention can be carried out in
either of two general ways: by introducing into the
patient a therapeutically effective amount of purified
human α-gal A obtained from cultured human cells
genetically modified to overexpress and secrete the
15 enzyme, or by introducing the overexpressing cell itself
into the patient. Techniques to accomplish the necessary
genetic modifications are discussed below, as are the
methods of purification, formulation, and treatment.

Example I.   Preparation and Use of Constructs Designed
20 to Deliver and Express α-Gal A
        Two expression plasmids, pXAG-16 and pXAG-28, were
constructed. These plasmids contain human α-gal A cDNA
encoding the 398 amino acids of the α-gal A enzyme
(without the α-gal A signal peptide); the human growth
25 hormone (hGH) signal peptide genomic DNA sequence, which
is interrupted by the first intron of the hGH gene; and
the 3' untranslated sequence (UTS) of the hGH gene, which
contains a signal for polyadenylation. Plasmid
pXAG-16 has the human cytomegalovirus immediate-early
30 (CMV IE) promoter and first intron (flanked by non-coding
exon sequences), while pXAG-28 is driven by the collagen
Iα2 promoter and also contains the β-actin gene's 5' UTS,
which contains the first intron of the β-actin gene.

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A. Cloning of the Complete α-Gal A
cDNA, and Construction of the α-Gal A
Expression Plasmid pXAG-16

The human α-gal cDNA was cloned from a human
5 fibroblast cDNA library that was constructed as follows.
Poly-A⁺ mRNA was isolated from total RNA, and cDNA
synthesis was performed using reagents for the
lambda ZapII® system according to the manufacturer's
instructions (Stratagene Inc., LaJolla, CA). Briefly,
10 "first strand" cDNA was generated by reverse
transcription in the presence of an oligo-dT primer
containing an internal XhoI restriction endonuclease
site. Following treatment with RNase H, the cDNA was
nick-translated with DNA polymerase I to generate double
15 stranded cDNA. This cDNA was made blunt-ended with
T4 DNA polymerase, and ligated to EcoRI adaptors. The
products of this ligation were treated with T4 DNA kinase
and digested with XhoI. The cDNA was fractionated by
Sephacryl-400® chromatography. Large and medium size
20 fractions were pooled and the cDNAs ligated to EcoRI and
XhoI-digested Lambda ZapII arms. The products of this
ligation were then packaged and titered. The primary
library had a titer of 1.2 x 10⁷ pfu/ml and an average
insert size of 925 bp.
25   A 210 bp probe from exon 7 of the human α-gal A
gene (Fig. 1, SEQ ID NO:19) was used to isolate the cDNA.
The probe itself was isolated from genomic DNA by the
polymerase chain reaction (PCR) using the following
oligonucleotides: 5'-CTGGGCTGTAGCTATGATAAAC-3' (Oligo 1;
30 SEQ ID NO:1) and 5'-TCTAGCTGAAGCAAAACAGTG-3' (Oligo 2;
SEQ ID NO:2). The PCR product was then used to screen
the fibroblast cDNA library, and positive clones were
isolated and further characterized. One positive clone,
phage 3A, was subjected to the lambda ZapII® system
35 excision protocol (Stratagene, Inc., La Jolla, CA),

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according to the manufacturer's instructions. This
procedure yielded plasmid pBSAG3A, which contains the α-
gal A cDNA sequence in the pBluescriptSK-™ plasmid
backbone. DNA sequencing revealed that this plasmid did
5 not contain the complete 5' end of the cDNA sequence.
Therefore, the 5' end was reconstructed using a PCR
fragment amplified from human genomic DNA. To accomplish
this, a 268 bp genomic DNA fragment (Fig. 2, SEQ ID
NO:20) was amplified using the following
10 oligonucleotides: 5'-ATTGGTCCGCCCCTGAGGT-3' (Oligo 3;
SEQ ID NO:3) and 5'-TGATGCAGGAATCTGGCTCT-3' (Oligo 4;
SEQ ID NO:4). This fragment was subcloned into a "TA"
cloning plasmid (Invitrogen Corp., San Diego, CA) to
generate plasmid pTAAGEI. Plasmid pBSAG3A, which
15 contains the majority of the α-gal A cDNA sequence, and
pTAAGEI, which contains the 5' end of the α-gal A cDNA,
were each digested with SacII and NcoI. The positions of
the relevant SacII and NcoI sites within the amplified
DNA fragment are shown in Fig. 2. The 0.2 kb SacII-NcoI
20 fragment from pTAAGEI was isolated and ligated to
equivalently digested pBSAG3A. This plasmid, pAGAL,
contains the complete α-gal A cDNA sequence, including
the sequence encoding the α-gal A signal peptide. The
cDNA was completely sequenced (shown in Fig. 3 including
25 the α-gal A signal peptide; SEQ ID NO: 18) and found to
be identical to the published sequence for the human α-
gal A cDNA (Genbank sequence HUMGALA).

        The plasmid pXAG-16 was constructed via several
intermediates, as follows. First, pAGAL was digested
30 with SacII and XhoI and blunt-ended. Second, the ends of
the complete α-gal A cDNA were ligated to XbaI linkers
and subcloned into XbaI digested pEF-BOS (Mizushima et
al., Nucl. Acids Res. 18:5322, 1990), creating pXAG-1.
This construct contains the human granulocyte-colony
35 stimulating factor (G-CSF) 3' UTS and the human

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elongation factor-1α (EF-1α) promoter flanking the cDNA
encoding α-gal A plus the α-gal A signal peptide, such
that the 5' end of the α-gal A cDNA is fused to the EF-1α
promoter. To create a construct with the CMV IE promoter

5 and first intron, the α-gal A cDNA and G-CSF 3' UTS were
removed from pXAG-1 as a two kb XbaI-BamHI fragment. The
fragment was blunt-ended, ligated to BamHI linkers, and
inserted into BamHI digested pCMVflpNeo (which was
constructed as described below). The orientation was

10 such that the 5' end of the α-gal A cDNA was fused to the
CMV IE promoter region.
        pCMVflpNeo was created as follows. A CMV IE gene
promoter fragment was amplified by PCR using CMV genomic
DNA as a template and the oligonucleotides:

15 5'-TTTTGGATCCCTCGAGGACATTGATTATTGACTAG-3' (SEQ ID NO:23)
and 5'-TTTTGGATCCCGTGTCAAGGACGGTGAC-3' (SEQ ID NO:24).
The resulting product (a 1.6 kb fragment) was digested
with BamHI, yielding a CMV promoter-containing fragment
with cohesive BamHI-digested ends. The neo expression

20 unit was isolated from plasmid pMC1neopA (Stratagene
Inc., La Jolla, CA) as a 1.1 kb XhoI-BamHI fragment. The
CMV promoter-containing and neo fragments were inserted
into a BamHI-, XhoI-digested plasmid (pUC12). Notably,
pCMVflpNeo contains the CMV IE promoter region, beginning

25 at nucleotide 546 and ending at nucleotide 2105 (of
Genbank sequence HS5MIEP), and the neomycin resistance
gene driven by the Herpes Simplex Virus (HSV) thymidine
kinase promoter (the TKneo gene) immediately 5' to the
CMV IE promoter fragment. The direction of transcription

30 of the neo gene is the same as that of the CMV promoter
fragment. This intermediate construct was called pXAG-4.
        To add the hGH 3' UTS, the GCSF 3' UTS was removed
from pXAG-4 as an XbaI-SmaI fragment and the ends of
pXAG-4 were made blunt. The hGH 3' UTS was removed from

35 pXGH5 (Selden et al., Mol. Cellular Biol. 6:3173-3179,

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1986) as a 0.6 kb SmaI-EcoRI fragment. After blunt-
ending this fragment, it was ligated into pXAG-4
immediately after the blunt-ended XbaI site of pXAG-4.
This intermediate was called pXAG-7. The TKneo fragment
5 was removed from this plasmid as a HindIII-ClaI fragment
and the ends of the plasmid were blunted by "filling-in"
with the Klenow fragment of DNA polymerase I. A neomycin
resistance gene driven by the SV40 early promoter was
ligated in as a blunted ClaI-BsmBI fragment from a digest
10 of pcDNeo (Chen et al., Mol. Cellular Biol. 7:2745-2752,
1987), placing the neo transcription unit in the same
orientation as the α-gal A transcription unit. This
intermediate was called pXAG-13.

        To complete pXAG-16, which has the 26 amino acid
15 hGH signal peptide coding sequence and first intron of
the hGH gene, a 2.0 kb EcoRI-BamHI fragment of pXAG-13
was first removed. This fragment included the α-gal A
cDNA and the hGH 3' UTS. This large fragment was
replaced with 3 fragments. The first fragment consisted
20 of a 0.3 kb PCR product of pXGH5, which contains the hGH
signal peptide coding sequence and includes the hGH first
intron sequence, from a synthetic BamHI site located just
upstream of the Kozak consensus sequence to the end of
the hGH signal peptide coding sequence. The following
25 oligonucleotides were used to amplify this fragment
(Fragment 1): 5'-TTTTGGATCCACCATGGCTA-3' (Oligo HGH101;
SEQ ID NO:5) and 5'-TTTTGCCGGCACTGCCCTCTTGAA-3'
(Oligo HGH102; SEQ ID NO:6). The second fragment
consisted of a 0.27 kb PCR product containing sequences
30 corresponding to the start of the cDNA encoding the 398
amino acid α-gal A enzyme (i.e., lacking the α-gal A
signal peptide) to the NheI site. The following
oligonucleotides were used to amplify this fragment
(Fragment 2): 5'-TTTTCAGCTGGACAATGGATTGGC-3' (Oligo AG10;
35 SEQ ID NO:7) and 5'-TTTTGCTAGCTGGCGAATCC-3' (Oligo AG11;

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SEQ ID NO:8). The third fragment consisted of the NheI-
EcoRI fragment of pXAG-7 containing the remaining α-gal A
sequence as well as the hGH 3' UTS (Fragment 3).

     Fragment 1 (digested with BamHI and NaeI),
5 Fragment 2 (digested with PvuII and NheI), and Fragment 3
were mixed with the 6.5 kb BamHI-EcoRI fragment of pXAG-
13 containing the neo gene and the CMV IE promoter and
ligated together to generate plasmid pXAG-16 (Figure 4).

     B. Construction of the α-Gal A
10   Expression Plasmid pXAG-28

     The human collagen Iα2 promoter was isolated for
use in the α-gal A expression construct pXAG-28 as
follows. A 408 bp PCR fragment of human genomic DNA
containing part of the human collagen Iα2 promoter was
15 isolated using the following oligonucleotides:
5'-TTTTGGATCCGTGTCCCATAGTGTTTCCAA-3'(Oligo 72;
SEQ ID NO:9) and 5'-TTTTGGATCCGCAGTCGTGGCCAGTACC-3'
(Oligo 73; SEQ ID NO:10).

     This fragment was used to screen a human leukocyte
20 library in EMBL3 (Clontech Inc., Palo Alto, CA). One
positive clone (phage 7H) containing a 3.8 kb EcoRI
fragment was isolated and cloned into pBSIISK+
(Stratagene Inc., La Jolla, CA) at the EcoRI site
(creating pBS/7H.2). An AvrII site was introduced in
25 pBSIISK+ by digesting with SpeI, which cleaves within the
pBSIISK+ polylinker, "filling-in" with the Klenow
fragment of DNA polymerase I, and inserting the
oligonucleotide 5'-CTAGTCCTAGGA-3' (SEQ ID NO:11). This
variant of pBSIISK+ was digested with BamHI and AvrII and
30 ligated to the 121 bp BamHI-AvrII fragment of the
original 408 bp collagen Iα2 promoter PCR fragment
described above, creating pBS/121COL.6.

     The plasmid pBS/121COL.6 was digested with XbaI,
which cleaves within the pBSIISK+ polylinker sequence,
35 "filled-in" with the Klenow fragment of DNA polymerase I,

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and digested with AvrII. The 3.8 kb BamHI-AvrII fragment
of pBS/7H.2 was isolated and the BamHI site made
blunt-ended by treatment with Klenow enzyme. The
fragment was then digested with AvrII and ligated to the
5 AvrII-digested vector, thus creating the collagen
promoter plasmid pBS/121bpCOL7H.18.

        Next the collagen promoter was fused to the 5' UTS
of the human β-actin gene, which contains the first
intron of the human β-actin gene. To isolate this
10 sequence, a 2 kb PCR fragment was isolated from human
genomic DNA using the following oligonucleotides:
5'-TTTTGAGCACAGAGCCTCGCCT-3' (Oligo BA1; SEQ ID NO:12)
and
5'-TTTTGGATCCGGTGAGCTGCGAGAATAGCC-3' (Oligo BA2;
15 SEQ ID NO:13).

        This fragment was digested with BamHI and BsiHKAI
to release a 0.8 kb fragment containing the β-actin
5' UTS and intron. A 3.6 kb SalI-SrfI fragment was then
isolated from the collagen promoter plasmid
20 pBS/121bpCOL7H.18 as follows. pBS/121bpCOL7H.18 was
partially digested with BamHI (the BamHI site lies at the
5' end of the collagen Iα2 promoter fragment), made
blunt-ended by treatment with the Klenow fragment, and
ligated to a SalI linker (5'-GGTCGACC-3'), thereby
25 placing a SalI site upsteam of the collagen Iα2 promoter.
This plasmid was then digested with SalI and SrfI (the
SrfI site lies 110 bp upstream of the collagen Iα2
promoter CAP site), and the 3.6 kb fragment was isolated.
The 0.8 and 3.6 kb fragments were combined with SalI -
30 and BamHI - digested pBSIISK- (Stratagene Inc.,
La Jolla, CA), and a fragment composed of the following
four oligonucleotides annealed together (forming a
fragment with a blunt end and a BsiHKAI end):
5'-GGGCCCCCAGCCCCAGCCCTCCCATTGGTGGAGGCCCTTTTGGAGGCACCCTAG
35 GGCCAGGAAACTTTTGCCGTAT-3' (Oligo COL-1; SEQ ID NO:14),

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5'-AAATAGGGCAGATCCGGGCTTTATTATTTTAGCACCACGGCCGCCGAGAC

CGCGTCCGCCCCGCGAGCA-3' (Oligo COL-2; SEQ ID NO:15),

5'-TGCCCTATTTATACGGCAAAAGTTTCCTGGCCCTAGGGTGCCTCCAAAAGGGC

CTCCACCAATGGGAGGGCTGGGGCTGGGGGCCC-3' (Oligo COL-3;

5   SEQ ID NO:16), and 5'-CGCGGGGCGGACGCGGTCTCGGCGGCCGTGGTGCT

AAAATAATAAAGCCCGGATC-3' (Oligo COL-4; SEQ ID NO:17).

These four oligonucleotides, when annealed, correspond to

the region beginning at the SrfI site of the collagen

promoter and continuing through the BsiHKAI site of the

10   β-actin promoter. The resulting plasmid was designated

pCOL/β-actin.

    To complete the construction of pXAG-28, the

SalI-BamHI fragment of pCOL/β-actin, containing the

collagen Iα2 promoter and β-actin 5' UTS, was isolated.

15 This fragment was ligated to two fragments from pXAG-16

(see Example 1A and Fig. 4):   (1) the 6.0 kb BamHI

fragment (containing the neo gene, plasmid backbone, the

cDNA encoding the 398 amino acid α-gal A enzyme, and the

hGH 3' UTS); and (2) the 0.3 kb BamHI-XhoI fragment

20 (which contains the SV40 poly A sequence from pcDneo).

pXAG-28 contains the human collagen Iα2 promoter fused to

the human β-actin 5' UTS, the hGH signal peptide (which

is interrupted by the hGH first intron), the cDNA

encoding the α-gal A enzyme, and the hGH 3' UTS. A map

25 of the completed expression construct pXAG-28 is shown in

Fig. 5.

        C.   Transfection and Selection of Fibroblasts
             Electroporated with α-Gal A Expression Plasmids

        In order to express α-gal A in fibroblasts,

30 secondary fibroblasts were cultured and transfected

according to published procedures (Selden et al.,

WO 93/09222).

        The plasmids pXAG-13, pXAG-16 and pXAG-28 were

transfected by electroporation into human foreskin

35 fibroblasts to generate stably transfected clonal cell

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strains, and the resulting α-gal A expression levels were monitored as described in Example ID. Secretion of α-gal A by normal foreskin fibroblasts is in the range of 2-10 units/10^6 cells/24 hours. In contrast, the
5 transfected fibroblasts displayed mean expression levels as shown in Table 1:

Table 1:   Mean α-gal A expression levels (+/- standard deviation)

---

10 pXAG-13:   420 +/- 344 U/10^6 cells/day
            N=26 clonal strains
            (range 3 - 1133 U/10^6 cells/day)

pXAG-16:   2,051 +/- 1253 U/10^6 cells/day
            N=24 clonal strains
15   (range 422 - 5200 U/10^6 cells/day)

pXAG-28:   141 +/- 131 U/10^6 cells/day
            N=38 clonal strains
            (range 20 - 616 U/10^6 cells/day)

These data show that all three expression
20 constructs are capable of increasing α-gal A expression many times that of nontransfected fibroblasts. Expression by fibroblasts stably transfected with pXAG-13, which encodes α-gal A linked to the α-gal A signal peptide, was substantially lower than expression by
25 fibroblasts transfected with pXAG-16, which differs only in that the signal peptide is the hGH signal peptide, the coding sequence of which is interrupted by the first intron of the hGH gene.

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Each time the transfected cells were passaged, the
secreted α-gal A activity was determined, the cells were
counted, and the cell density was calculated. Based on
the number of cells harvested and the time allowed for
5 secretion of α-gal A, the specific expression rate of α-
gal A was determined and is reported in Tables 2 and 3 as
secreted units (of α-gal A) per 10^6 cells per 24 hour
period. Cell strains desirable for gene therapy or for
use in generation of material for purification of α-gal A
10 should display stable growth and expression over several
passages. Data from the cell strains shown in Tables 2
and 3, which were stably transfected with the α-gal A
expression construct pXAG-16, illustrate the fact that α-
gal A expression is stably maintained during serial
15 passage.

Table 2:   Growth and Expression of BRS-11 Cells
            Containing the α-Gal A Expression Construct
            pXAG-16.

BRS-11		
Passage	Expression (units/10^6 cells/24 hr)	Cell Density (cells/cm^2)
13	2601	4.80×10^4
14	1616	4.40×10^4
15	3595	4.40×10^4

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Table 3:   Growth and Expression of HF503-242 Cells
            Containing the α-Gal A Expression Construct
            pXAG-16.

HF503-242		
Passage	Expression (units/10^6 cells/24hr)	Cell Density (cells/cm^2)
5	4069	2.80 x 10^4
6	7585	3.55 x 10^4
7	5034	2.48 x 10^4

D.   Quantification of α-Gal A Expression

The activity of α-gal A activity was measured
using the water-soluble substrate 4-methylumbelliferyl-α-
D-galactopyranoside (4-MUF-gal; Research Products, Inc.)
by a modification of the protocol described by Ioannou et
al. (J. Cell Biol. 119:1137-1150, 1992). The substrate
was dissolved in substrate buffer (0.1 M citrate-
phosphate,
pH 4.6) to a concentration of 1.69 mg/ml (5 mM).
Typically, 10 μl of culture supernatant was added to 75
μl of the substrate solution. The tubes were covered and
allowed to incubate in a 37°C waterbath for 60 minutes.
At the end of the incubation period, 2 ml of glycine-
carbonate buffer (130 mM glycine, 83 mM sodium carbonate,
at pH 10.6), were used to stop the reaction. The
relative fluorescence of each sample was measured using a
model TK0100 fluorometer (Hoefer Scientific Instruments)
which has a fixed excitation wavelength of 365 nm and
detects a fixed emission wavelength of 460 nm. The

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readings of the samples were compared to standards
prepared from a 1 μM stock of methylumbelliferone (Sigma
Chemical Co.), and the amount of hydrolyzed substrate was
calculated. The activity of α-gal A is expressed in
5 units; one unit of α-gal A activity is equivalent to one
nanomole of substrate hydrolyzed per hour at 37°C. Cell
expression data were generally expressed as units of α-
gal A activity secreted/10^6 cells/24 hours. This assay
was also used to measure the amount of α-gal activity in
10 cell lysates and in samples from various α-gal
purification steps, as discussed below.

Example II. Purification of α-Gal A from the Conditioned
Medium of Stably Transfected Human Cell Strains
         Examples IIA-IIE illustrate that α-gal A may be
15 purified to near-homogeneity from the conditioned medium
of cultured human cell strains that have been stably
transfected to produce the enzyme.
         A. Use of Butyl Sepharose® Chromatography
         as a First Step in the Purification of α-Gal A
20   Cold conditioned medium (1.34 liters) was
clarified by centrifugation and filtered through a
0.45 μm cellulose acetate filter using glass fiber
prefilters. While stirring, the pH of the cold, filtered
medium was adjusted to 5.6 by the dropwise addition of
25 1 N HCl, and ammonium sulfate was added to a final
concentration of 0.66 M by the dropwise addition of a
stock solution (room temperature) of 3.9 M ultrapure
ammonium sulfate. The medium was stirred for an
additional 5 minutes at 4°C, filtered as before, and
30 applied to a Butyl Sepharose® 4 Fast Flow column (81 ml
column volume, 2.5 x 16.5 cm; Pharmacia, Uppsala, Sweden)
that had been equilibrated in 10 mM MES-Tris, pH 5.6,
containing 0.66 M ammonium sulfate (buffer A). The
chromatography was performed at 4°C on a Gradi-Frac™

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System (Pharmacia, Uppsala, Sweden) equipped with in-line
UV (280 nm) and conductivity monitors for assessing total
protein and salt concentration, respectively. After
sample application at a flow rate of 10 ml/min, the
5 column was washed with 10 column volumes of buffer A.
The α-gal A was eluted from the Butyl Sepharose® column
with a 14 column volume linear gradient from buffer A
(containing ammonium sulfate) to 10 mM MES-Tris, pH 5.6
(no ammonium sulfate). Fractions were assayed for
10 α-gal A activity by the 4-MUF-gal assay, and those
containing appreciable enzyme activity were pooled. As
seen in Fig. 6 and the purification summary (Table 3),
this step removes approximately 99% of the contaminating
protein (pre-column sample=8.14 g total protein; post-
15 column sample=0.0638 g total protein).

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Table 4:   Purification of α-Gal A from the Conditioned
           Medium of Stably Transfected Human Fibroblasts

Purification Step	Volume (ml)	α-Gal A Activity (x 10^6 Units)	Total Protein (mg)	Specific Activity (x 10^6 Units /mg)	Fold Purification (Cumulative)	Percent Recovery
Culture supernatant	1340	14.6	8140	0.0018	=1	=100
Butyl Sepharose®	417	14.1	63.8	0.221	123	96.6
Heparin Sepharose®	134	12.1	14.6	0.829	436	82.9
Hydroxyapatite	47	9.73	4.46	2.18	1220	66.6
Q Sepharose®	31.5	8.91	3.31	2.69	1503	61.0
Superdex® 200	10	8.58	2.93	2.92	1634	59.0

B. Use of Heparin Sepharose® Chromatography
    as a Step for Purification of α-Gal A

The Butyl Sepharose® column peak fractions were
dialyzed at 4°C against (4 liters) of 10 mM MES-Tris,
pH 5.6 (changed once). The conductivity of the dialysate
was adjusted to 1.0 mMHO at 4°C by addition of H₂O or
NaCl as necessary. Afterward, the sample was applied to
a column of Heparin Sepharose® 6 Fast Flow (Pharmacia,
Uppsala, Sweden; 29 ml column volume, 2.5 x 6 cm) that
had been equilibrated in 10 mM MES-Tris, pH 5.6,
containing 9 mM NaCl (buffer B). This was done at 4°C at
a flow rate of 10 ml/min. In-line UV (280 nm) and

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conductivity monitors measured total protein and salt
concentration. After the sample was applied, the column
was washed with 10 column volumes of buffer B followed by
a 3 column volume linear gradient to 8% buffer C/92%
5 buffer B (where buffer C is 10 mM MES-Tris, pH 5.6,
containing 250 mM NaCl) and a 10 column volume wash with
8% buffer C. This was followed by elution of α-gal A
with a 1.5 column volume linear gradient to 29% buffer C
and a subsequent 10 column volume linear gradient to
10 35% buffer C. Fractions were assayed for α-gal A
activity, and those containing appreciable activity were
pooled.

           C. Use of Hydroxyapatite
           Chromatography as a Step for
15   Purification of α-Gal A
           The heparin pool was filtered and applied
directly to a column of Ceramic Hydroxyapatite HC (40 μm;
American International Chemical, Natick, MA; 12 ml column
volume, 1.5 x 6.8 cm) that had been equilibrated in 1 mM
20 sodium phosphate, pH 6.0 (buffer D). The chromatography
was performed at room temperature on a hybrid Gradi-
Frac™/FPLC® System (Pharmacia, Uppsala, Sweden) equipped
with in-line UV (280 nm) and conductivity monitors.
After the sample was applied (5 ml/min), the column was
25 washed with 10 column volumes of buffer D. The α-gal A
was eluted with a 7 column volume linear gradient to 42%
buffer E/58% buffer D (where buffer E is 250 mM sodium
phosphate, pH 6.0) followed by a 10 column volume
gradient to 52% buffer E. Fractions were assayed for α-
30 gal A activity, and the fractions containing appreciable
activity were pooled.

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D. Use of Q Sepharose® Anion
Exchange Chromatography as a Step
for Purification of α-Gal A

The hydroxyapatite pool was diluted

5 approximately 1.5 fold with H_2O to a final conductivity
of 3.4-3.6 mMHO at room temperature. After filtering,
the sample was applied to a column of Q Sepharose® HP
(Pharmacia, Uppsala, Sweden; 5.1 ml column volume,
1.5 x 2.9 cm) equilibrated in 10% buffer G/90% buffer F,

10 where buffer F is 25 M sodium phosphate, pH 6.0, and
buffer G is 25 mM sodium phosphate, pH 6.0, 250 mM NaCl.
The chromotography was performed at room temperature on
the Gradi-Frac™/FPLC® hybrid system (Pharmacia, Uppsala,
Sweden), and total protein and salt concentrations were

15 monitored by the in-line monitors. The sample was
applied at a flow rate of 5 ml/min, then the following
steps were performed: (1) a 5 column volume wash at
10% buffer G, (2) a 7 column volume wash at 12% buffer G,
(3) a 3 column volume linear gradient to 50% buffer G,

20 (4) a 10 column volume linear gradient to 53% buffer G,
(5) a 3 column volume gradient to 100% buffer G, and
(6) a 10 column volume wash at 100% buffer G. The α-
gal A eluted primarily during steps 3 and 4. Fractions
containing appreciable activity were pooled (the

25 "Q pool").

E. Use of Superdex®-200 Gel
Filtration Chromatography as a Step
for Purification of α-Gal A

The Q pool was concentrated approximately 5-

30 fold using Centriprep®-10 centrifugal concentrator units
(Amicon, Beverly, MA), and applied to a column of
Superdex® 200 (Pharmacia, Uppsala, Sweden; 189 ml column
volume, 1.6 x 94 cm). The column was equilibrated and
eluted with 25 mM sodium phosphate, pH 6.0, containing

35 150 mM NaCl. The chromatography was performed on an

FPLC® system (Pharmacia, Uppsala, Sweden) at room
temperature using an in-line UV monitor (280 nm) to
follow elution of the protein. The volume of the sample
applied to the column was ≤ 2 ml, the flow rate was 0.5
5 ml/min, and the fraction size was 2 ml. Multiple column
runs were performed; fractions were assayed for α-gal A
activity and fractions containing appreciable activity
were pooled.

      The pooled fractions from the Superdex® 200
10 column were concentrated using Centriprep-10 units,
aliquoted, snap-frozen, and stored at -80°C for short
periods of time. A summary of this example of α-gal A
purification is shown in Table 3. The final yield of α-
gal A was 59% of the starting material activity, and the
15 specific activity of the purified product was
2.92 x 10^6 units/mg protein. The resulting product
showed a high level of purity after electrophoresis under
reducing conditions on a 4-15% SDS-polyacrylamide gel,
which was subsequently silver-stained.

20 <u>Example III. Formulation and Storage of Purified α-Gal A</u>
      Highly purified α-gal A is not stable for
extended periods of time when stored as a dilute solution
of purified protein (≤1 mg protein/ml). Therefore, a
formulation was devised to improve stability during
25 prolonged storage, i.e. storage lasting several weeks to
at least several months. The purified enzyme was
concentrated to at least 1 mg/ml using a centrifugal
concentrator (in enzyme buffer consisting of 25 mM sodium
phosphate (pH 6.0) and 150 mM NaCl). Human serum albumin
30 (HSA; Buminate®, Baxter-Hyland) was added to a final
concentration of 2.5 mg/ml. The protein solution was
then sterile filtered using a 0.2 μm cellulose acetate
filter (Schleicher and Schuell) attached to a syringe.
The α-gal A solution was dispensed into sterile, pyrogen-

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free glass vials, sealed with a Teflon cap, snap-frozen, and stored at -20°C.

Stability of the α-gal A activity was evaluated over a three month period using the 4-MUF-gal assay. The data presented in Table 5 demonstrate that there was no loss of enzyme activity over the test period. The acidic pH of the formulation (< 6.5) is critical to the stability of the highly purified enzyme.

Table 5: Stability of Formulated α-Gal A at -20°C

Sample	Specific Activity (Units/mg total protein)
time 0	2.24×10^6 +/- 0.33×10^6
week 1	2.40×10^6 +/- 0.25×10^6
week 2	2.42×10^6 +/- 0.21×10^6
week 3	2.37×10^6 +/- 0.05×10^6
month 1	2.39×10^6 +/- 0.16×10^6
month 2	2.31×10^6 +/- 0.26×10^6
month 3	2.29×10^6 +/- 0.17×10^6

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Example IV. α-Gal A Produced by Human Cell Strains is
Suitable for Treatment of α-Gal A Deficiency

       The structural and functional properties of
purified human α-gal A prepared in accordance with the
5 invention were investigated in order to demonstrate that
the DNA molecules described herein and the corresponding
expressed glycoproteins produced by transfected human
cell strains can be used in gene or enzyme replacement
therapies, respectively.

10   A. Size of α-Gal A Produced by Stably
Transfected   Human Cells in Culture

       The molecular mass of α-gal A was estimated by
MALDI-TOF mass spectrometry. These results demonstrate
that the molecular mass of the dimer is 102,353 Da, while
15 that of the monomer is 51,002 Da. The expected molecular
mass of the monomer, based on amino acid composition, is
45,400 Da. Therefore, it can be inferred that the
carbohydrate content of the enzyme accounts for up to
5,600 Da of the molecular weight.

20   The results of standard amino acid analysis
performed on the purified protein are consistent with the
conclusion that the protein produced by transfected human
cells is identical to the protein purified from human
tissues at the amino acid level.

25   B. N-Terminal Processing of α-Gal A
Produced by Stably Transfected Human Cells

       The human α-gal A cDNA nucleotide sequence
encodes 429 amino acids. The 31 N-terminal amino acids
constitute a signal peptide sequence, which is cleaved as
30 the nascent protein transits the endoplasmic reticulum
membrane (LeDonne et al., Arch. Biochem. Biophys.
224:186, 1983; Lemansky et al., J. Biol. Chem. 262:2062,
1987). In order to confirm that α-gal A is properly
processed when associated with an heterologous signal
35 peptide sequence (for example, the human growth hormone

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signal sequence) and expressed in transfected human
fibroblasts, ten N-terminal amino acids of the secreted
protein were microsequenced. Samples were
electrophoresed by SDS-PAGE and transferred to
5 ProBlott® (ABI, Foster City, CA) using a 10 mM CAPS
(pH 11.0), 10% methanol buffer system. The protein on
the ProBlott® was visualized by Coomassie staining, and
an appropriately sized (50 kDa) band was excised. N-
terminal sequence was obtained using an Applied
10 Biosystems pulse-liquid phase amino acid sequenator that
performs automated Edman degradation. The N-terminal
sequence obtained, LDNGLARTPT (SEQ ID NO:28), is
consistent with proper cleavage of the signal peptide and
matches the N-terminal sequence predicted for the
15 secreted protein.

   C. C-Terminal Amino Acid of α-Gal A
Produced by Stably Transfected Human Cells
   The C-terminal amino acid residue of secreted
α-gal A produced in accordance with the invention was
20 identified using an automated Hewlett Packard C-terminal
sequencer. The results indicated a leucine residue at
the C-terminus, which agrees with the C-terminal amino
acid predicted by the DNA sequence.

   D. Carbohydrate Modification of α-Gal A
25   Produced by Stably Transfected Human Cells
   The glycosylation pattern of α-gal A produced
in accordance with the invention was also evaluated.
Proper glycosylation is important for optimal *in vivo*
activity of α-gal A; α-gal A expressed in non-
30 glycosylating systems is inactive or unstable
(Hantzopolous et al., Gene 57:159, 1987). Glycosylation
is also important for the internalization of α-gal A into
the desired target cells, and affects the circulating
half-life of the enzyme *in vivo*. On each subunit of α-
35 gal A there are four sites available for addition of

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asparagine-linked carbohydrate chains, of which only
three are occupied (Desnick et al., In <u>The Metabolic and
Molecular Bases of Inherited Disease</u>, pp 2741-2780,
McGraw Hill, New York, 1995).

5   A sample of α-gal A produced by stably
transfected cells was treated with neuraminidase, which
is isolated from *A. urafaciens*, (Boehringer-Mannheim,
Indianapolis, IN) to remove sialic acid. This reaction
was performed by treating 5 μg of α-gal A overnight with
10   10 mU of neuraminidase at room temperature in a total
volume of 10 μl of acetate buffered saline (ABS, 20 mM
sodium acetate, pH. 5.2, 150 mM NaCl).

          Purified α-gal A produced by stably transfected
cells was also dephosphorylated using alkaline
15   phosphatase (calf intestinal alkaline phosphatase,
Boehringer-Mannheim, Indianapolis, IN), by treating 5 μg
of α-gal A overnight at room temperature with 15 U of
alkaline phosphatase in ABS (pH raised to 7.5 with 1 M
Tris).

20   The samples were analyzed by Western blot with
an α-gal A-specific antibody. The antibody used was a
rabbit polyclonal anti-peptide antibody, which was
produced using a peptide representing amino acids 68-81
of α-gal A as an immunogen. Following transfer of the
25   protein to PVDF (Millipore, Bedford, MA), the membrane
was probed with a 1:2000 dilution of the anti-serum in
2.5% blotto (non-fat dry milk in 20 mM Tris-HCl, pH 7.5,
0.05% Tween-20). This was followed by detection with
goat anti-rabbit IgG conjugated to horseradish peroxidase
30   (Organon Teknika/Cappel, Durham, NC; 1:5000 dilution) and
reagents of the ECL chemiluminescence kit (Amersham,
Arlington Heights, IN).

          Treatment of α-gal A with neuraminidase results
in a slight shift in molecular mass (approximately 1500-
35   2000 Da or 4-6 sialic acids/monomer), suggesting that

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there is extensive modification of α-gal A with sialic
acid. For reference, the plasma form of α-gal A has 5-6
sialic acid residues per monomer, and the placental form
has 0.5-1.0 sialic acid residues per monomer (Bishop
5 et al., J. Biol. Chem. 256:1307, 1981).

Another method used to examine the sialic acid
and mannose-6-phosphate modifications of α-gal A was
isoelectric focusing (IEF), where the samples are
separated on the basis of their isoelectric point (pI) or
10 net charge. Thus, removal of charged residues such as
sialic acid or phosphate from α-gal A would be expected
to alter the mobility of the protein in the IEF system.

To perform the IEF experiment, samples of α-
gal A produced in accordance with the invention were
15 treated with neuraminidase and alkaline phosphatase,
mixed 1:1 with 2X Novex sample buffer (with 8 M urea,
pH 3.0-7.0), and loaded onto a 6 M urea IEF gel
(5.5% polyacrylamide) made using Pharmalyte® (Pharmacia,
Uppsala, Sweden; pH 3.0-6.5; Pharmalyte® 4-6.5 and 2.5-
20 5.5, 0.25 ml each per gel). Isoelectric point standards
(Bio-Rad) were also included. Following electrophoresis,
the gel was transferred to PVDF, and Western blot
analysis performed as described above.

The α-gal A produced by stably transfected
25 human fibroblasts consisted of three major isoforms with
a pI range of approximately 4.4-4.65. These values are
similar to the pIs of the plasma and splenic forms of
α-gal A (Bishop et al., J. Biol. Chem. 256:1307, 1981).
Neuraminidase treatment of the enzyme increased the pI of
30 all three isoforms, indicating that all were modified to
some extent by sialic acid. These data suggest that the
α-gal A produced by stably transfected human cells should
have a desirable plasma half-life, indicating that this
material is well suited for pharmacologic use. Further,
35 treatment of neuraminidase-treated α-gal A with alkaline

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phosphatase further increased the pI of a portion of the
protein to approximately 5.0-5.1, indicating that the
enzyme bears one or more mannose-6-phosphate residues.
This modification is significant in that it is required
5 for efficient internalization of α-gal A by the target
cells.

                    E. Specific Activity of α-gal A
                    Purified from Stably Transfected Fibroblasts
                    The potency or specific activity of purified
10 α-gal A is calculated by measuring both the catalytic
activity of the enzyme (with the 4-MUF-gal assay), and
the protein concentration. The protein concentration can
be determined by any standard method, such as with the
BCA system (Pierce), or by measuring the absorbance at
15 280 nm and using the mg/ml extinction coefficient of 2.3
(determined from amino acid analysis) to calculate the
value. Using these techniques, the specific activity of
α-gal A purified from the conditioned medium of
transfected human fibroblasts is 2.2-2.9 x 10^6 units/mg
20 of protein, which is comparable to the specific activity
of α-gal A that is purified from human tissues (Bishop
et al., J. Biol. Chem. 256:1301, 1981).

                    F. Mannose or Mannose-6-Phosphate Mediated
                    Internalization of α-Gal A
25   In order for the α-gal A produced by stably
transfected cells to be an effective therapeutic agent
for α-gal A deficiencies, the enzyme must be internalized
by the affected cells. α-Gal A is not active at
physiological pH levels, and is unlikely to be effective
30 in the blood or interstitial fluids. It metabolizes
accumulated lipid substrates optimally only when
internalized in the acidic environment of the lysosome.
This internalization is mediated by the binding of α-
gal A to mannose-6-phosphate (M6P) receptors, which are
35 expressed on the cell surface and deliver the enzyme to

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the lysosome via the endocytic pathway. The M6P receptor
is ubiquitously expressed; most somatic cells express it
to some extent. The mannose receptor, which is specific
for exposed mannose residues on glycoproteins, is less
5 prevalent. The latter receptors are generally found only
on macrophage and macrophage-like cells, and provide an
additional means of α-gal A entry into these cell types.
       In order to demonstrate M6P-mediated
internalization of α-gal A, skin fibroblasts from a Fabry
10 disease patient (NIGMS Human Genetic Mutant Cell
Repository) were cultured overnight in the presence of
increasing concentrations of purified α-gal A of the
invention. Some of the samples contained 5 mM soluble
M6P, which competitively inhibits binding to, and as a
15 result, internalization by, the mannose-6 phosphate
receptor. Other samples contained 30 μg/ml mannan, which
inhibits binding to, and as a result, internalization by,
the mannose receptor. Following incubation, the cells
were washed and harvested by scraping into lysis buffer
20 (10 mM Tris, pH 7.2, 100 mM NaCl, 5 mM EDTA, 2 mM
Pefabloc™ (Boehringer-Mannheim, Indianapolis, IN) and
1% NP-40). The lysed samples were then assayed for
protein concentration and α-gal A activity. The results
are expressed as units of α-gal A activity/mg cell
25 protein. The Fabry cells internalized α-gal A in a dose-
dependent manner (Fig. 7). This internalization was
inhibited by mannose-6 phosphate, but there was no
inhibition with mannan. Therefore, internalization of
α-gal A in Fabry fibroblasts is mediated by the mannose-6
30 phosphate receptor, but not by the mannose receptor.
       α-gal A is also internalized in vitro by
endothelial cells, important target cells for the
treatment of Fabry disease. Human umbilical vein
endothelial cells (HUVECs) were cultured overnight with
35 7500 units of α-gal A; some of the wells contained M6P.

After the incubation period, cells were harvested and
assayed for α-gal A as described above. The cells
incubated with α-gal A only had enzyme levels almost 10-
fold above those of control (no incubation with α-gal A)
5   cells. M6P inhibited the intracellular accumulation of
α-gal A, suggesting that the internalization of α-gal A
by HUVECs is mediated by the M6P receptor. Thus, the
human α-gal A of the invention is internalized by
clinically relevant cells.
10   Few cultured human cell lines are known to
express the mannose receptor. However, a mouse
macrophage-like cell line (J774.E) which bears mannose
receptors but few if any mannose 6-phosphate receptors
can be used to determine whether purified α-gal A of the
15   invention is internalized via the mannose receptor
(Diment et al., J. Leukocyte Biol. 42:485-490, 1987).
J774.E cells were cultured overnight in the presence of
10,000 units/ml α-gal A. Selected samples also contained
2 mM M6P, and others contained 100 μg/ml mannan. The
20   cells were washed and harvested as described above, and
the total protein and α-gal A activity of each sample was
determined. The results are shown in Table 5. M6P does
not inhibit the uptake of α-gal A by these cells, while
mannan decreases the accumulated α-gal A levels by 75%.
25   Thus, the α-gal A of the invention may be internalized by
the mannose receptor in cell types that express this
particular cell surface receptor.

Table 6.   Internalization of α-Gal A by J774.E Cells.

α-Gal A Activity (units/mg total protein)

	No additions	+ α-gal A	+ α-gal A, + M6P	+α-gal A, + mannan
J774.E	409±25	6444±554	6297±674	1654±323

[mannan] = 100 μg/ml

5 [M6P] = 2 mM or 660 μg/ml

These experiments demonstrate that the α-gal A
produced by stably transfected human cells may be
internalized by cells via the mannose or mannose-6-
phosphate receptor.

10   G.   Correction of Fabry Fibroblasts by Human
Fibroblasts Expressing α-Gal A
For gene therapy, an implant of autologous
cells producing α-gal A must produce the enzyme in a form
modified appropriately to "correct" the α-gal A

15 deficiency in target cells. To assess the effect of α-
gal A production by transfected human fibroblasts on
Fabry cells, fibroblasts harvested from Fabry disease
patients (NIGMS Human Genetics Mutant Cell Repository)
were co-cultured with an α-gal A-producing cell strain

20 (BRS-11) in Transwells® (Costar, Cambridge, MA). The
experimental scheme is depicted in Fig. 8. Fabry cells
were cultured in 12-well tissue culture dishes, some of
which contained inserts (Transwells®, 0.4 μm pore size)
having a surface on which cells can be grown. The growth

25 matrix of the insert is porous and allows macromolecules
to pass from the upper to the lower milieu. One set of
inserts contained normal human foreskin (HF) fibroblasts,

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which secrete minimal levels of α-gal A, while another
set contained the stably transfected human fibroblast
strain, BRS-11, which secretes large amounts of α-gal A.
In the wells co-cultured with α-gal A-producing cells, α-
5   gal A can enter the medium bathing the Fabry cells, and
potentially be internalized by the Fabry cells.

        The data in Table 7 show that Fabry cells
internalized the secreted α-gal A. The intracellular
levels of α-gal A were monitored for three days. Those
10 cells cultured alone (no insert) or in the presence of
non-transfected foreskin fibroblasts (HF insert) had very
low intracellular levels of α-gal A activity. The Fabry
cells cultured with the α-gal A-producing (BRS-11 insert)
cells, however, exhibited enzyme levels similar to those
15 of normal cells by the end of Day 2 (normal fibroblasts
have 25-80 units α-gal A/mg protein). That the
correction is attributable to α-gal A taken up via the
M6P receptor is demonstrated by its inhibition with
mannose-6-phosphate (BRS-11 insert + M6P).

20 Table 7. Correction of Fabry Fibroblasts by Human
            Fibroblasts Expressing α-Gal A

            α-Gal A Activity (units/mg total protein)

Time	no insert	HF insert	BRS-11 insert	BRS-11 insert+M6P
Day 1	2 ± 1	2 ± 1	13 ± 1	4 ± 1
Day 2	2 ± 1	2 ± 1	40 ± 11	6 ± 2
Day 3	2 ± 1	5 ± 1	85 ± 1	9 ± 1

H. Utility of Other Cell Types

        Other cell types can be used in the method
described herein. The cells can be obtained from a
30 variety of tissues and include all cell types that can be

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maintained in culture. For example, primary and
secondary cells that can be transfected by the present
method include human fibroblasts, keratinocytes,
epithelial cells (e.g., mammary or intestinal epithelial

5 cells), endothelial cells, glial cells, neural cells,
formed elements of the blood (e.g., lymphocytes and bone
marrow cells), muscle cells, and precursors of these
somatic cell types. Fibroblasts are of particular
interest. Primary cells are preferably obtained from the

10 individual to whom the transfected primary or secondary
cells are to be administered, so that they will not be
rejected by the patient's immune system. However, if
proper attention is paid to avoiding or suppressing
immunorejection (as described below), cells of a human

15 donor other than the patient can be used as well. This
would permit use of cells from an standardized,
established, stably transfected cell line in all
patients.

I. Administration of α-Gal A-Expressing Cells

20   The cells described above may be introduced
into an individual, through various standardized routes
of administration, so that they will reside in, for
example, the renal subcapsule, a subcutaneous
compartment, the central nervous system, the intrathecal

25 space, the liver, the intraperiotoneal cavity, or within
a muscle. The cells may also be injected intravenously
or intra-arterially so that they circulate within the
individual's bloodstream. Once implanted in the
individual, the transfected cells will produce and.

30 secrete the therapeutic product, glycosylated human α-gal
A.

The number of genetically modified cells that
will be introduced into the individual will vary, but can
be determined by skilled artisans. The age, weight, sex,

35 and general physical condition of each patient, as well

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as the volume of distribution, the half-life and
bioavailability of the enzyme, and the *in vivo*
productivity of the genetically modified cells, will be
among the primary considerations in determining dosage
5   and route of administration. Typically, between one
million and one billion cells will be used, with
expression levels ranging from 100-100,000 units per 10^6
cells per day. If necessary, the procedure may be
repeated or modified until the desired result, for
10 example, relief from the symptoms associated with Fabry
disease, is achieved.

As described above, the cells used will
generally be patient-specific, i.e., obtained from the
individual to whom the transfected primary or secondary
15 cells are to be administered, so that they will not be
rejected by the patient's immune system. If, however,
this scenario is not possible or desirable, cells may be
obtained from another individual, genetically modified as
described herein, and implanted into the patient who is
20 suffering from α-gal A deficiency.

The use of cells from an individual other than
the recipient might require administration of an
immunosuppressant, alteration of histocompatibility
antigens, or use of a barrier device to prevent rejection
25 of the implanted cells. The barrier device will be made
of a material (e.g., a membrane such as XM-50 from
Amicon, Beverly, MA) that permits the secreted product to
pass into the recipient's circulation or tissues, but
prevents contact between the implanted cells and the
30 recipient's immune system, and thus prevents an immune
response to (and possible rejection of) the cells by the
recipient. For further guidance regarding gene therapy,
see Selden et al. (WO 93/09222).

The cells may alternatively be embedded in a
35 matrix or gel material, such as described in co-owned

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U.S.S.N. 08/548/002, which describes the use of hybrid
matrix implants, or in Jain et al. (PCT application
WO 95/19430), which describes macroencapsulation of
secretory cells in a hydrophilic gel material (each of
5   which is hereby incorporated by reference).

J. Pharmaceutical Formulation for Conventional
Administration of α-Gal A Protein

The α-gal A protein that is expressed and
secreted by stably transfected (or otherwise genetically
10 modified) human cells and purified as described herein
may be administered to individuals who produce
insufficient or defective α-gal A protein. The protein
may be administered in a pharmaceutically acceptable
carrier, at a pH below 6.5, e.g. in a formulation as
15 described in Example III. Examples of excipients which
may be included with the formulation are buffers such as
citrate buffer, phosphate buffer, acetate buffer, and
bicarbonate buffer, amino acids, urea, alcohols, ascorbic
acid, phospholipids, proteins, such as serum albumin and
20 gelatin, EDTA, sodium chloride, liposomes,
polyvinylpyrollidone, mannitol, sorbitol, glycerol,
propylene glycol and polyethylene glycol (e.g., PEG-4000,
PEG-6000). The route of administration may be, for
example, intravenous, intra-arterial, subcutaneous,
25 intraperitoneal, intracerebral, intramuscular,
intrapulmonary, or transmucosal. The route of
administration and the amount of protein delivered will
be determined by factors that are well within the ability
of skilled artisans to assess. Furthermore, skilled
30 artisans are aware that the route of administration and
dosage of a therapeutic protein may be varied for a given
patient until a therapeutic dosage level is obtained.
Typically, doses of α-gal A of 0.01-100 mg/kg of body
weight will be administered. It is expected that

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regularly repeated doses of the protein will be necessary over the life of the patient.

K. Treatment of Other Conditions
Caused by Enzyme Deficiencies

5   It is likely that other conditions caused by deficiencies in lysosomal storage enzymes other than α-gal A will be amenable to treatment by methods comparable to those described herein. In these cases, DNA which encodes a functional form of the deficient

10 enzyme would be substituted for the DNA encoding α-gal A in the expression constructs disclosed herein. Examples of enzyme deficiency syndromes that have been identified, and that may be amenable to treatment as described herein, are shown in Table 8. The information in this

15 Table is taken from E. Neufeld (Ann. Rev. Biochem. 60:257-280, 1991), which is hereby incorporated by reference.

Table 8. Summary of Lysosomal Storage Disorders

_____

20 Disorder   Primary deficiency
                                   [secondary deficiency]

Disorders of sphingolipid degradation

Fabry disease   α-galactosidase

Farber disease   ceramidase

25 Gaucher disease   glucocerebrosidase

G$_{M1}$ gangliosidosis   β-galactosidase

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G$_{M2}$ gangliosidoses   β-hexosaminidase, α-
subunit
   Tay-Sachs disease   [hexosaminidase A]

   Sandhoff disease   β-hexosaminidase, β-
5 subunit
                        [hexosaminidases A and B]

   Activator deficiency   G$_{M2}$ activator
Krabbe disease   galactosylceramidase

Metachromatic leukodystrophy
10   enzyme-deficient form   arylsulfatase A
   activator-deficient form   sulfatide
activator/saposin

   Mucolipidosis IV   primary defect unknown
                        [ganglioside sialidase]

15 Multiple sulfatase deficiency   primary defect unknown
                        [deficiency of all
sulfatases]

   Niemann-Pick disease   sphingomyelinase

   Schindler disease   α-N-
20 acetylgalactosaminidase

Disorders of glycoprotein degradation

   Aspartylglycosaminuria   aspartylglycosaminidase

   Fucosidosis   α-L-fucosidase

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| Galactosialidosis protein/cathepsin | protective |
| sialidase] | [β-galactosidase and |

5 α-Mannosidosis   α-mannosidase

β-Mannosidosis   β-mannosidase

Sialidosis   sialidase

<u>Disorders of glycosaminoglycan degradation</u>

Hunter syndrome   iduronate sulfatase

10 Hurler and Scheie syndromes   α-L-iduronidase

Maroteaux-Lamy syndrome   GalNAc 4-
sulfatase/arylsulfatase

Morquio syndrome

    A-subtype   Gal 6-sulfatase

15   B-subtype   β-galactosidase

Sanfilippo syndrome
    A-subtype   heparan N-sulfatase

    B-subtype   α-N-acetylglucosaminidase

    C-subtype   AcetylCoA: glucosamine

20   N-acetyltransferase

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D-subtype	GlcNAc 6-sulfatase
Sly syndrome	β-glucuronidase

Other single enzyme deficiency disorders

Pompe disease (glycogenosis II)	α-glucosidase
5 Wolman disease	acid lipase

Disorders of lysosomal enzyme biosynthesis

I-cell disease and pseudoHurler	6-phospho-N-
acetylglucosamine	
	transferase
10 polydystrophy	[mislocalization of many
lysosomal	
	enzymes]

Disorders of lysosmal membrane transport

Cystinosis	cystine transport
15 Sialic storage and Salla disease	sialic acid transport

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V. Other Embodiments

The invention described herein has been exemplified in part by methods of treatment that employ cells which express a particular gene product following
5 transfection, i.e., after introduction of a construct encoding the gene product and having regulatory elements that control expression of the coding sequence. These methods may also be carried out using cells that have been genetically modified by other procedures, including
10 gene targeting and gene activation (see Treco et al. (WO 95/31560, herein incorporated by reference; see also Selden et al. WO 93/09222).

The hGH signal peptide can be used with heterologous proteins other than α-gal A, to increase the
15 level of expression and secretion of the heterologous protein. Examples of such proteins include α-1 antitrypsin, antithrombin III, apolipoprotein E, apolipoprotein A-1, blood clotting factors V, VII, VIII, IX, X, and XIII, bone growth factor-2, bone growth
20 factor-7, calcitonin, catalytic antibodies, DNAse, erythropoietin, FSH-β, globins, glucagon, glucocerebrosidase, G-CSF, GM-CSF, growth hormone, immune response modifiers, immunoglobulins, insulin, insulinotropin, insulin-like growth factors,
25 interferon-β, interferon-β nerve growth factors, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6, interleukin-11, interleukin-12, IL-2 receptor, IL-1 receptor antagonists, low density lipoprotein receptor, M-CSF, parathyroid hormone, protein
30 kinase C, soluble CD4, superoxide dismutase, tissue plasminogen activator, TGF-β, tumor necrosis factor, TSHβ, tyrosine hydroxylase, and urokinase.

Other embodiments are within the following claims.

50

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Transkaryotic Therapies, Inc.

   (ii) TITLE OF INVENTION: THERAPY FOR α-GALACTOSIDASE A
       DEFICIENCY

  (iii) NUMBER OF SEQUENCES: 28

   (iv) CORRESPONDENCE ADDRESS:
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    (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

   (vi) CURRENT APPLICATION DATA:
      (A) APPLICATION NUMBER:
      (B) FILING DATE:
      (C) CLASSIFICATION:

  (vii) PRIOR APPLICATION DATA:
      (A) APPLICATION NUMBER: 08/712,614
      (B) FILING DATE: 13-SEP-1996
      (C) CLASSIFICATION:

 (viii) ATTORNEY/AGENT INFORMATION:
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      (B) REGISTRATION NUMBER: 34,819
      (C) REFERENCE/DOCKET NUMBER: 07236/003WO1

   (ix) TELECOMMUNICATION INFORMATION:
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      (B) TELEFAX: 617/542-8906
      (C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 22 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGGGCTGTA GCTATGATAA AC   22

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:

51

          (A) LENGTH: 21 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCTAGCTGAA GCAAAACAGT G   21

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 19 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTGGTCCGC CCCTGAGGT   19

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGATGCAGGA ATCTGGCTCT   20

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTTGGATCC ACCATGGCTA   20

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 24 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTTTGCCGGC ACTGCCCTCT TGAA   24

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 24 base pairs
          (B) TYPE: nucleic acid

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```
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTTTCAGCTG GACAATGGAT TGGC                                         24

(2) INFORMATION FOR SEQ ID NO:8:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 20 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTTTGCTAGC TGGCGAATCC                                              20

(2) INFORMATION FOR SEQ ID NO:9:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTTGGATCC GTGTCCCATA GTGTTTCCAA                                   30

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 28 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTTGGATCC GCAGTCGTGG CCAGTACC                                     28

(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTAGTCCTAG GA                                                      12

(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 22 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
```

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTTTGAGCAC AGAGCCTCGC CT   22

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 30 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTTTGGATCC GGTGAGCTGC GAGAATAGCC   30

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 76 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGCCCCCAG CCCCAGCCCT CCCATTGGTG GAGGCCCTTT TGGAGGCACC CTAGGGCCAG   60

GAAACTTTTG CCGTAT   76

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 69 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAATAGGGCA GATCCGGGCT TTATTATTTT AGCACCACGG CCGCCGAGAC CGCGTCCGCC   60

CCGCGAGCA   69

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 86 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGCCCTATTT ATACGGCAAA AGTTTCCTGG CCCTAGGGTG CCTCCAAAAG GGCCTCCACC   60

AATGGGAGGG CTGGGGCTGG GGGCCC   86

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:

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```
        (A) LENGTH: 55 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGCGGGGCGG ACGCGGTCTC GGCGGCCGTG GTGCTAAAAT AATAAAGCCC GGATC            55

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1343 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCGCGGGAAA TTTATGCTGT CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC       60

ATCTGGGCTG CGCGCTTGCG CTTCGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG      120

CTAGAGCACT GGACAATGGA TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC      180

GCTTCATGTG CAACCTTGAC TGCCAGGAAG AGCCAGATTC CTGCATCAGT GAGAAGCTCT      240

TCATGGAGAT GGCAGAGCTC ATGGTCTCAG AAGGCTGGAA GGATGCAGGT TATGAGTACC      300

TCTGCATTGA TGACTGTTGG ATGGCTCCCC AAAGAGATTC AGAAGGCAGA CTTCAGGCAG      360

ACCCTCAGCG CTTTCCTCAT GGGATTCGCC AGCTAGCTAA TTATGTTCAC AGCAAAGGAC      420

TGAAGCTAGG GATTTATGCA GATGTTGGAA ATAAAACCTG CGCAGGCTTC CCTGGGAGTT      480

TTGGATACTA CGACATTGAT GCCCAGACCT TTGCTGACTG GGGAGTAGAT CTGCTAAAAT      540

TTGATGGTTG TTACTGTGAC AGTTTGGAAA ATTTGGCAGA TGGTTATAAG CACATGTCCT      600

TGGCCCTGAA TAGGACTGGC AGAAGCATTG TGTACTCCTG TGAGTGGCCT CTTTATATGT      660

GGCCCTTTCA AAAGCCCAAT TATACAGAAA TCCGACAGTA CTGCAATCAC TGGCGAAATT      720

TTGCTGACAT TGATGATTCC TGGAAAAGTA TAAAGAGTAT CTTGGACTGG ACATCTTTTA      780

ACCAGGAGAG AATTGTTGAT GTTGCTGGAC CAGGGGGTTG GAATGACCCA GATATGTTAG      840

TGATTGGCAA CTTTGGCCTC AGCTGGAATC AGCAAGTAAC TCAGATGGCC CTCTGGGCTA      900

TCATGGCTGC TCCTTTATTC ATGTCTAATG ACCTCCGACA CATCAGCCCT CAAGCCAAAG      960

CTCTCCTTCA GGATAAGGAC GTAATTGCCA TCAATCAGGA CCCCTTGGGC AAGCAAGGGT     1020

ACCAGCTTAG ACAGGGAGAC AACTTTGAAG TGTGGGAACG ACCTCTCTCA GGCTTAGCCT     1080

GGGCTGTAGC TATGATAAAC CGGCAGGAGA TTGGTGGACC TCGCTCTTAT ACCATCGCAG     1140

TTGCTTCCCT GGGTAAAGGA GTGGCCTGTA ATCCTGCCTG CTTCATCACA CAGCTCCTCC     1200

CTGTGAAAAG GAAGCTAGGG TTCTATGAAT GGACTTCAAG GTTAAGAAGT CACATAAATC     1260

CCACAGGCAC TGTTTTGCTT CAGCTAGAAA ATACAATGCA GATGTCATTA AAAGACTTAC     1320
```

55

TTTAAAAAAA AAAAAAACTC GAG   1343

(2) INFORMATION FOR SEQ ID NO:19:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 210 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTGGGCTGTA GCTATGATAA ACCGGCAGGA GATTGGTGGA CCTCGCTCTT ATACCATCGC   60

AGTTGCTTCC CTGGGTAAAG GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT   120

CCCTGTGAAA AGGAAGCTAG GGTTCTATGA ATGGACTTCA AGGTTAAGAA GTCACATAAA   180

TCCCACAGGC ACTGTTTTGC TTCAGCTAGA   210

(2) INFORMATION FOR SEQ ID NO:20:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 268 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATTGGTCCGC CCCTGAGGTT AATCTTAAAA GCCCAGGTTA CCCGCGGAAA TTTATGCTGT   60

CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC ATCTGGGCTG CGCGCTTGCG   120

CTTCGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG CTAGAGCACT GGACAATGGA   180

TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC GCTTCATGTG CAACCTTGAC   240

TGCCAGGAAG AGCCAGATTC CTGCATCA   268

(2) INFORMATION FOR SEQ ID NO:21:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 26 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

    Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu
    1   5   10   15

    Cys Leu Pro Trp Leu Gln Glu Gly Ser Ala
                20   25

(2) INFORMATION FOR SEQ ID NO:22:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 78 base pairs

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```
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATGGCTACAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG GCCTGCTCTG CCTGCCCTGG      60

CTTCAAGAGG GCAGTGCC                                                    78

(2) INFORMATION FOR SEQ ID NO:23:

      (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 35 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear


      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTTTGGATCC CTCGAGGACA TTGATTATTG ACTAG                                 35

(2) INFORMATION FOR SEQ ID NO:24:

      (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 28 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTTTGGATCC CGTGTCAAGG ACGGTGAC                                         28

(2) INFORMATION FOR SEQ ID NO:25:

      (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 1197 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTGGACAATG GATTGGCAAG GACGCCTACC ATGGGCTGGC TGCACTGGGA GCGCTTCATG      60

TGCAACCTTG ACTGCCAGGA AGAGCCAGAT TCCTGCATCA GTGAGAAGCT CTTCATGGAG     120

ATGGCAGAGC TCATGGTCTC AGAAGGCTGG AAGGATGCAG GTTATGAGTA CCTCTGCATT     180

GATGACTGTT GGATGGCTCC CCAAAGAGAT TCAGAAGGCA GACTTCAGGC AGACCCTCAG     240

CGCTTTCCTC ATGGGATTCG CCAGCTAGCT AATTATGTTC ACAGCAAAGG ACTGAAGCTA     300

GGGATTTATG CAGATGTTGG AAATAAAACC TGCGCAGGCT TCCCTGGGAG TTTTGGATAC     360

TACGACATTG ATGCCCAGAC CTTTGCTGAC TGGGGAGTAG ATCTGCTAAA ATTTGATGGT     420

TGTTACTGTG ACAGTTTGGA AAATTTGGCA GATGGTTATA AGCACATGTC CTTGGCCCTG     480
```

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```
AATAGGACTG GCAGAAGCAT TGTGTACTCC TGTGAGTGGC CTCTTTATAT GTGGCCCTTT      540

CAAAAGCCCA ATTATACAGA AATCCGACAG TACTGCAATC ACTGGCGAAA TTTTGCTGAC      600

ATTGATGATT CCTGGAAAAG TATAAAGAGT ATCTTGGACT GGACATCTTT TAACCAGGAG      660

AGAATTGTTG ATGTTGCTGG ACCAGGGGGT TGGAATGACC CAGATATGTT AGTGATTGGC      720

AACTTTGGCC TCAGCTGGAA TCAGCAAGTA ACTCAGATGG CCCTCTGGGC TATCATGGCT      780

GCTCCTTTAT TCATGTCTAA·TGACCTCCGA CACATCAGCC CTCAAGCCAA AGCTCTCCTT      840

CAGGATAAGG ACGTAATTGC CATCAATCAG GACCCCTTGG GCAAGCAAGG GTACCAGCTT      900

AGACAGGGAG ACAACTTTGA AGTGTGGGAA CGACCTCTCT CAGGCTTAGC CTGGGCTGTA      960

GCTATGATAA ACCGGCAGGA GATTGGTGGA CCTCGCTCTT ATACCATCGC AGTTGCTTCC     1020

CTGGGTAAAG GAGTGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT CCCTGTGAAA     1080

AGGAAGCTAG GGTTCTATGA ATGGACTTCA AGGTTAAGAA GTCACATAAA TCCCACAGGC     1140

ACTGTTTTGC TTCAGCTAGA·AAATACAATG CAGATGTCAT TAAAAGACTT ACTTTAA        1197
```

(2) INFORMATION FOR SEQ ID NO:26:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 398 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: peptide

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```
Leu Asp Asn Gly Leu Ala Arg Thr Pro Thr Met Gly Trp Leu His Trp
1               5                   10                  15

Glu Arg Phe Met Cys Asn Leu Asp Cys Gln Glu Glu Pro Asp Ser Cys
            20                  25                  30

Ile Ser Glu Lys Leu Phe Met Glu Met Ala Glu Leu Met Val Ser Glu
        35                  40                  45

Gly Trp Lys Asp Ala Gly Tyr Glu Tyr Leu Cys Ile Asp Asp Cys Trp
    50                  55                  60

Met Ala Pro Gln Arg Asp Ser Glu Gly Arg Leu Gln Ala Asp Pro Gln
65                  70                  75                  80

Arg Phe Pro His Gly Ile Arg Gln Leu Ala Asn Tyr Val His Ser Lys
            85                  90                  95

Gly Leu Lys Leu Gly Ile Tyr Ala Asp Val Gly Asn Lys Thr Cys Ala
            100                 105                 110

Gly Phe Pro Gly Ser Phe Gly Tyr Tyr Asp Ile Asp Ala Gln Thr Phe
            115                 120                 125

Ala Asp Trp Gly Val Asp Leu Leu Lys Phe Asp Gly Cys Tyr Cys Asp
    130                 135                 140
```

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```
Ser Leu Glu Asn Leu Ala Asp Gly Tyr Lys His Met Ser Leu Ala Leu
145              150              155                  160

Asn Arg Thr Gly Arg Ser Ile Val Tyr Ser Cys Glu Trp Pro Leu Tyr
                 165              170              175

Met Trp Pro Phe Gln Lys Pro Asn Tyr Thr Glu Ile Arg Gln Tyr Cys
             180              185              190

Asn His Trp Arg Asn Phe Ala Asp Ile Asp Asp Ser Trp Lys Ser Ile
         195              200              205

Lys Ser Ile Leu Asp Trp Thr Ser Phe Asn Gln Glu Arg Ile Val Asp
    210              215              220

Val Ala Gly Pro Gly Gly Trp Asn Asp Pro Asp Met Leu Val Ile Gly
225              230              235                  240

Asn Phe Gly Leu Ser Trp Asn Gln Gln Val Thr Gln Met Ala Leu Trp
             245              250              255

Ala Ile Met Ala Ala Pro Leu Phe Met Ser Asn Asp Leu Arg His Ile
             260              265              270

Ser Pro Gln Ala Lys Ala Leu Leu Gln Asp Lys Asp Val Ile Ala Ile
         275              280              285

Asn Gln Asp Pro Leu Gly Lys Gln Gly Tyr Gln Leu Arg Gln Gly Asp
    290              295              300

Asn Phe Glu Val Trp Glu Arg Pro Leu Ser Gly Leu Ala Trp Ala Val
305              310              315                  320

Ala Met Ile Asn Arg Gln Glu Ile Gly Gly Pro Arg Ser Tyr Thr Ile
             325              330              335

Ala Val Ala Ser Leu Gly Lys Gly Val Ala Cys Asn Pro Ala Cys Phe
             340              345              350

Ile Thr Gln Leu Leu Pro Val Lys Arg Lys Leu Gly Phe Tyr Glu Trp
         355              360              365

Thr Ser Arg Leu Arg Ser His Ile Asn Pro Thr Gly Thr Val Leu Leu
    370              375              380

Gln Leu Glu Asn Thr Met Gln Met Ser Leu Lys Asp Leu Leu
385              390              395
```

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 338 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
ATGGCTACAG GTAAGCGCCC CTAAAATCCC TTTGGGCACA ATGTGTCCTG AGGGGAGAGG      60

CAGCGACCTG TAGATGGGAC GGGGGCACTA ACCCTCAGGT TTGGGGCTTC TGAATGTGAG     120
```

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TATCGCCATG TAAGCCCAGT ATTTGGCCAA TCTCAGAAAG CTCCTGGTCC CTGGAGGGAT   180

GGAGAGAGAA AAACAAACAG CTCCTGGAGC AGGGAGAGTG CTGGCCTCTT GCTCTCCGGC   240

TCCCTCTGTT GCCCTCTGGT TTCTCCCCAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG   300

GCCTGCTCTG CCTGCCCTGG CTTCAAGAGG GCAGTGCC   338

(2) INFORMATION FOR SEQ ID NO:28:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 10 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: not relevant
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: peptide

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

      Leu Asp Asn Gly Leu Ala Arg Thr Pro Thr
      1   5   10

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What is claimed is:

1. A method of treatment comprising identifying a
patient suspected of having an α-galactosidase A
deficiency, and introducing into the patient a human cell
5 genetically modified to overexpress and secrete human α-
gal A.

2. The method of claim 1, wherein the cell has
been transfected *in vitro* with a DNA molecule having a
coding sequence encoding a polypeptide comprising human
10 α-gal A (SEQ ID NO:26).

3. The method of claim 2, wherein the polypeptide
further comprises an heterologous signal peptide.

4. The method of claim 3, wherein the
heterologous signal peptide is the human growth hormone
15 (hGH) signal peptide (SEQ ID NO:21).

5. The method of claim 3, wherein the DNA
molecule comprises an intron within the sequence encoding
the signal peptide.

6. The method of claim 3, wherein the DNA
20 molecule comprises an untranslated sequence of at least
6 nucleotides lying immediately 3' to the termination
codon of the coding sequence, said untranslated sequence
comprising a polyadenylation site.

7. A DNA molecule comprising
25   a first sequence encoding the hGH signal peptide
(SEQ ID NO:21), said first sequence comprising an intron;
and,

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    linked to the 3' end of the first sequence, a
second sequence encoding a polypeptide comprising human
α-gal A.

    8. The DNA molecule of claim 7, further
5 comprising a 3' untranslated sequence comprising a
polyadenylation site.

    9. An expression construct comprising the DNA
molecule of claim 7 and suitable for expression in a
human cell.

10   10. A cultured human cell containing a DNA
molecule that (a) encodes a polypeptide comprising human
α-gal A linked to an heterologous signal peptide, and
(b) permits expression of the polypeptide in the cell.

    11. The cell of claim 10, said cell being a
15 fibroblast.

    12. The cell of claim 10, wherein said cell is
selected from the group consisting of an epithelial cell,
an endothelial cell, a bone marrow cell, a glial cell, a
hepatocyte, a keratinocyte, a myocyte, a neuron, or a
20 precursor of these cell types.

    13. The cell of claim 10, wherein said signal
peptide is the hGH signal peptide (SEQ ID NO:21).

    14. A clonal cell strain consisting essentially
of a plurality of cultured human cells transfected with a
25 DNA molecule that (a) encodes a polypeptide comprising
human α-gal A linked to an heterologous signal peptide,
and (b) permits overexpression of the polypeptide in the
cells.

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15.   The clonal cell strain of claim 14, said
cells being fibroblasts.

16.   A clonal cell line consisting essentially of
a plurality of immortalized human cells transfected with
5   a DNA molecule that (a) encodes a polypeptide comprising
human α-gal A linked to an heterologous signal peptide,
and (b) permits overexpression of the polypeptide in the
cells.

17.   The clonal cell line of claim 16, wherein
10   said cell is selected from the group consisting of a
Bowes melanoma cell, a Daudi cell, a HeLa cell, an HL-60
cell, an HT1080 cell, a Jurkat cell, a KB carcinoma cell,
a K-562 leukemia cell, an MCF-7 breast cancer cell, a
MOLT-4 cell, a Namalwa cell, a Raji cell, an RPMI 8226
15   cell, a U-937 cell, a WI-38VA13 (subline 2R4) cell, and a
2780AD ovarian carcinoma cell.

18.   A protein comprising (a) the hGH signal
peptide (SEQ ID NO:21) linked by a peptide bond to (b)
human α-gal A.

20   19.   A process for making glycosylated human
α-gal A, comprising
        culturing the cell of claim 11 under conditions
permitting expression of human α-gal A from said DNA
molecule and secretion of glycosylated human α-gal A into
25   the culture medium of the cell; and
        isolating glycosylated human α-gal A from the
culture medium.

20.   Purified glycosylated human α-gal A made by
the process of claim 19.

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21. A process for purifying human α-gal A from a sample, comprising a first chromatography step wherein the sample is passed over a hydrophobic interaction resin.

5   22. The process of claim 21, wherein the functional moiety on the resin comprises a butyl group.

23. A process for making glycosylated human α-gal A, comprising
          culturing the cell of claim 16 under conditions
10   permitting expression of human α-gal A from said DNA molecule and secretion of glycosylated human α-gal A into the culture medium of the cell; and
          isolating glycosylated human α-gal A from the culture medium.

15   24. The process of claim 21, further comprising the step of passing the sample over a second resin selected from the group consisting of an immobilized heparin resin, hydroxyapatite, an anion exchange resin, and a size exclusion resin.

20   25. A method of treatment comprising
          identifying a patient suspected of having an α-gal A deficiency, and
          administering to the patient the purified glycosylated human α-gal A of claim 20.

25   26. A therapeutic composition comprising pharmaceutically acceptable purified human α-gal A having N-linked mannose-6-phosphate in a pharmaceutically acceptable carrier.

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27. A therapeutic composition comprising the
purified human α-gal A of claim 20 and a pharmaceutically
acceptable excipient.

28. The therapeutic composition of claim 27,
5 formulated at pH 6.5 or lower.

29. The therapeutic composition of claim 28,
wherein the pharmaceutically acceptable excipient is
human serum albumin.

30. A DNA molecule encoding a protein comprising
10 the signal peptide of hGH (SEQ ID NO:21) linked to a
polypeptide other than hGH.

31. The DNA molecule of claim 30, wherein the DNA
molecule comprises an intron within the sequence encoding
the signal peptide.

15   32. A cultured human cell containing a DNA
molecule that (a) encodes a polypeptide comprising human
α-gal A, and (b) permits overexpression of the
polypeptide in the cell.

33. A process for making glycosylated human
20 α-gal A, comprising culturing a human cell containing a
DNA molecule that encodes a polypeptide comprising human
α-gal A under conditions permitting expression of human
α-gal A from said DNA molecule.

34. The therapeutic composition of claim 26
25 further comprising a pharmaceutically acceptable
excipient.

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35. A process for purifying human α-gal A from a sample comprising passing said sample over a hydrophobic interaction resin comprising a butyl group as a functional moiety.

CTGGGCTGTAGCTATGATAAACCGGCAGGA

GATTGGTGGACCTCGCTCTTATACCATCGCA

GTTGCTTCCCTGGGTAAAGGAGTGGCCTGTA

ATCCTGCCTGCTTCATCACACAGCTCCTCCCT

GTGAAAAGGAAGCTAGGGTTCTATGAATGGA

CTTCAAGGTTAAGAAGTCACATAAATCCCAC

AGGCACTGTTTTGCTTCAGCTAGA

FIG. 1

ATTGGTCCGCCCCTGAGGTTAATCTTAAAAG

SacII
CCCAGGTTACCCGCGGAAATTTATGCTGTC

CGGTCACCGTGACAATGCAGCTGAGGAACC

CAGAACTACATCTGGGCTGCGCGCTTGCGCT

TCGCTTCCTGGCCCTCGTTTCCTGGGACATC

CCTGGGGCTAGAGCACTGGACAATGGATTG

NcoI
GCAAGGACGCCTACCATGGGCTGGCTGCAC

TGGGAGCGCTTCATGTGCAACCTTGACTGCC

AGGAAGAGCCAGATTCCTGCATCA

FIG. 2

```
   1 CCGCGGGAAA TTTATGCTGT CCGGTCACCG TGACAATGCA GCTGAGGAAC CCAGAACTAC
  61 ATCTGGGCTG CGCGCTTGCG CTTCGCTTCC TGGCCCTCGT TTCCTGGGAC ATCCCTGGGG
 121 CTAGAGCACT GGACAATGGA TTGGCAAGGA CGCCTACCAT GGGCTGGCTG CACTGGGAGC
 181 GCTTCATGTG CAACCTTGAC TGCCAGGAAG AGCCAGATTC CTGCATCAGT GAGAAGCTCT
 241 TCATGGAGAT GGCAGAGCTC ATGGTCTCAG AAGGCTGGAA GGATGCAGGT TATGAGTACC
 301 TCTGCATTGA TGACTGTTGG ATGGCTCCCC AAAGAGATTC AGAAGGCAGA CTTCAGGCAG
 361 ACCCTCAGCG CTTTCCTCAT GGGATTCGCC AGCTAGCTAA TTATGTTCAC AGCAAAGGAC
 421 TGAAGCTAGG GATTTATGCA GATGTTGGAA ATAAAACCTG CGCAGGCTTC CCTGGGAGTT
 481 TTGGATACTA CGACATTGAT GCCCAGACCT TTGCTGACTG GGAGTAGAT CTGCTAAAAT
 541 TTGATGGTTG TTACTGTGAC AGTTTGGAAA ATTTGGCAGA TGGTTATAAG CACATGTCCT
 601 TGGCCCTGAA TAGGACTGGC AGAAGCATTG TGTACTCCTG TGAGTGGCCT CTTTATATGT
 661 GGCCCTTTCA AAAGCCCAAT TATACAGAAA TCCGACAGTA CTGCAATCAC TGGCGAAATT
 721 TTGCTGACAT TGATGATTCC TGGAAAAGTA TAAAGAGTAT CTTGGACTGG ACATCTTTTA
 781 ACCAGGAGAG AATTGTTGAT GTTGCTGGAC CAGGGGGTTG GAATGACCCA GATATGTTAG
 841 TGATTGGCAA CTTTGGCCTC AGCTGGAATC AGCAAGTAAC TCAGATGGCC CTCTGGGCTA
 901 TCATGGCTGC TCCTTTATTC ATGTCTAATG ACCTCCGACA CATCAGCCCT CAAGCCAAAG
 961 CTCTCCTTCA GGATAAGGAC GTAATTGCCA TCAATCAGGA CCCCTTGGGC AAGCAAGGGT
1021 ACCAGCTTAG ACAGGAGAC AACTTTGAAG TGTGGGAACG ACCTCTCTCA GGCTTAGCCT
1081 GGGCTGTAGC TATGATAAAC CGGCAGGAGA TTGGTGGACC TCGCTCTTAT ACCATCGCAG
1141 TTGCTTCCCT GGGTAAAGGA GTGGCCTGTA ATCCTGCCTG CTTCATCACA CAGCTCCTCC
1201 CTGTGAAAAG GAAGCTAGGG TTCTATGAAT GGACTTCAAG GTTAAGAAGT CACATAAATC
1261 CCACAGGCAC TGTTTTGCTT CAGCTAGAAA ATACAATGCA GATGTCATTA AAAGACTTAC
1321 TTTAAAAAAA AAAAAAACTC GAG
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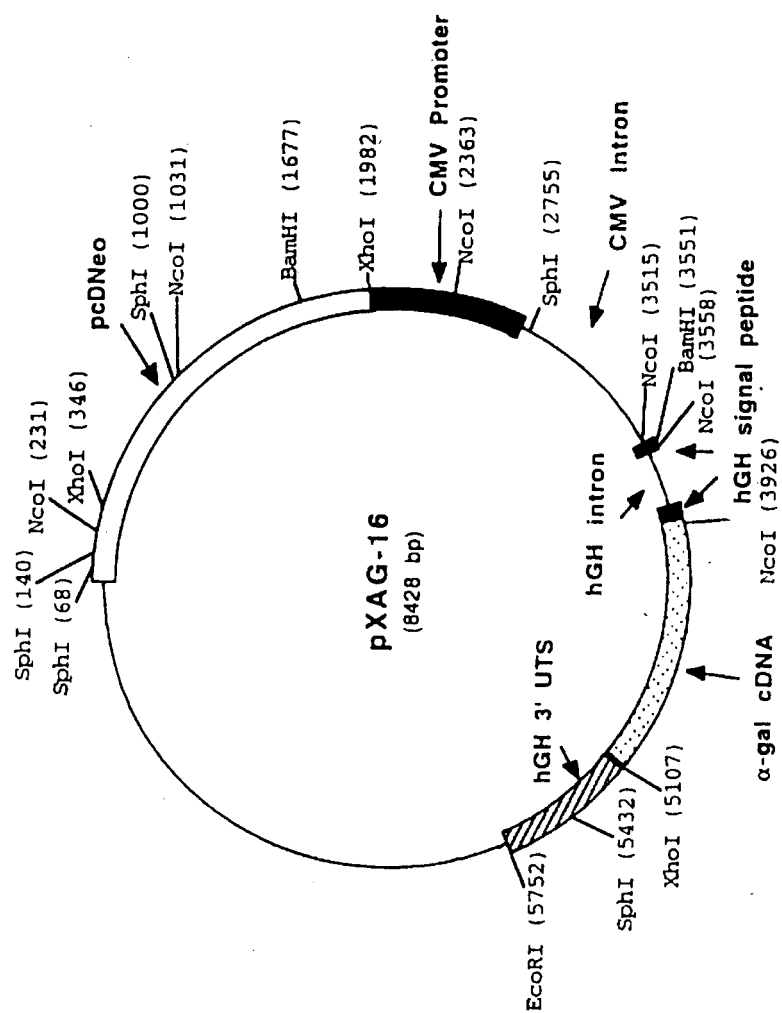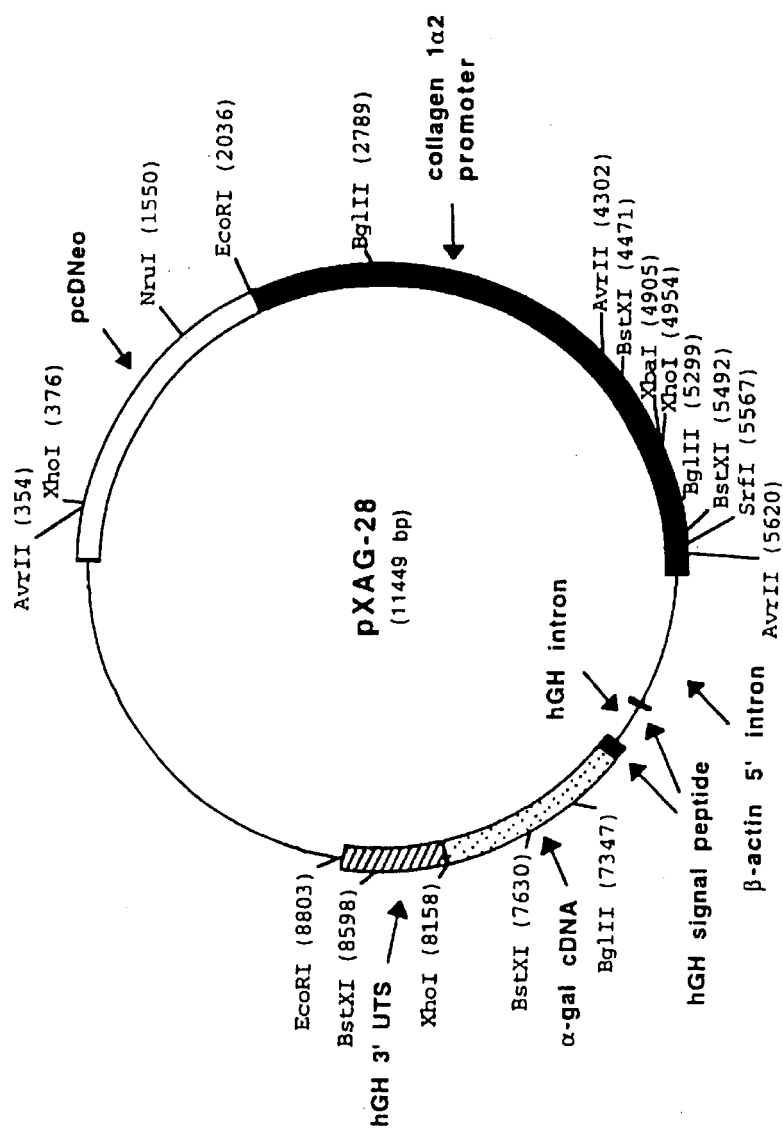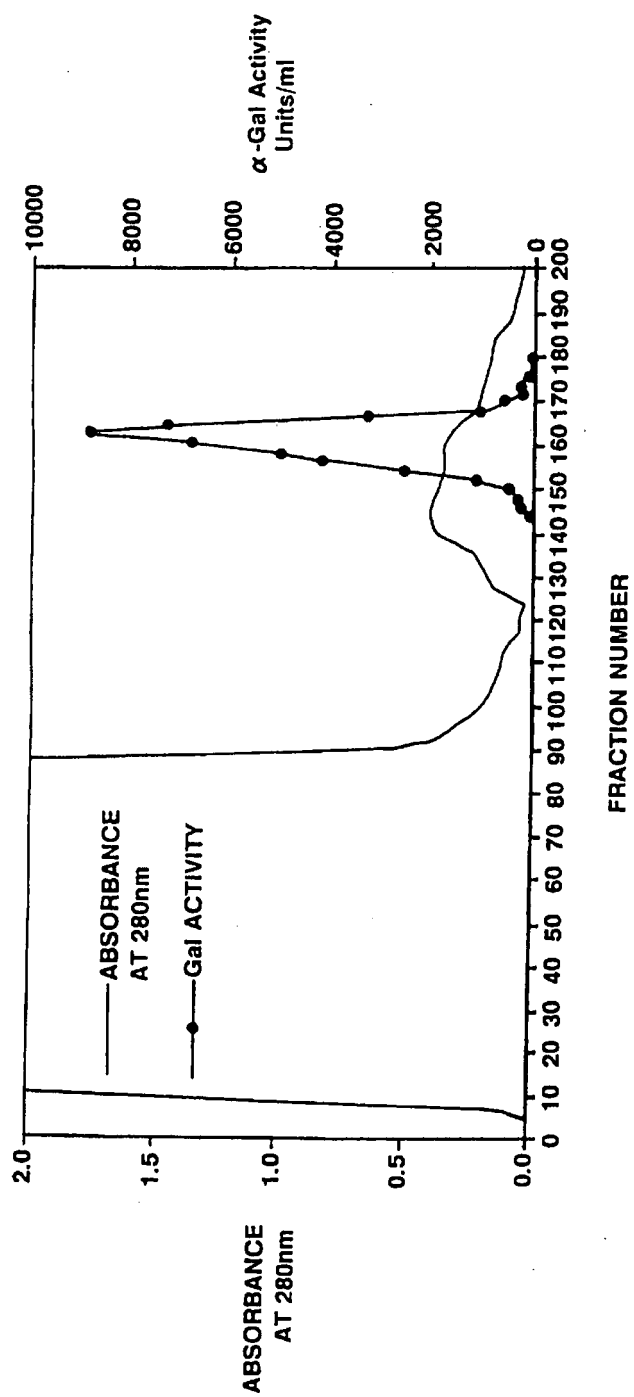
FIG. 3
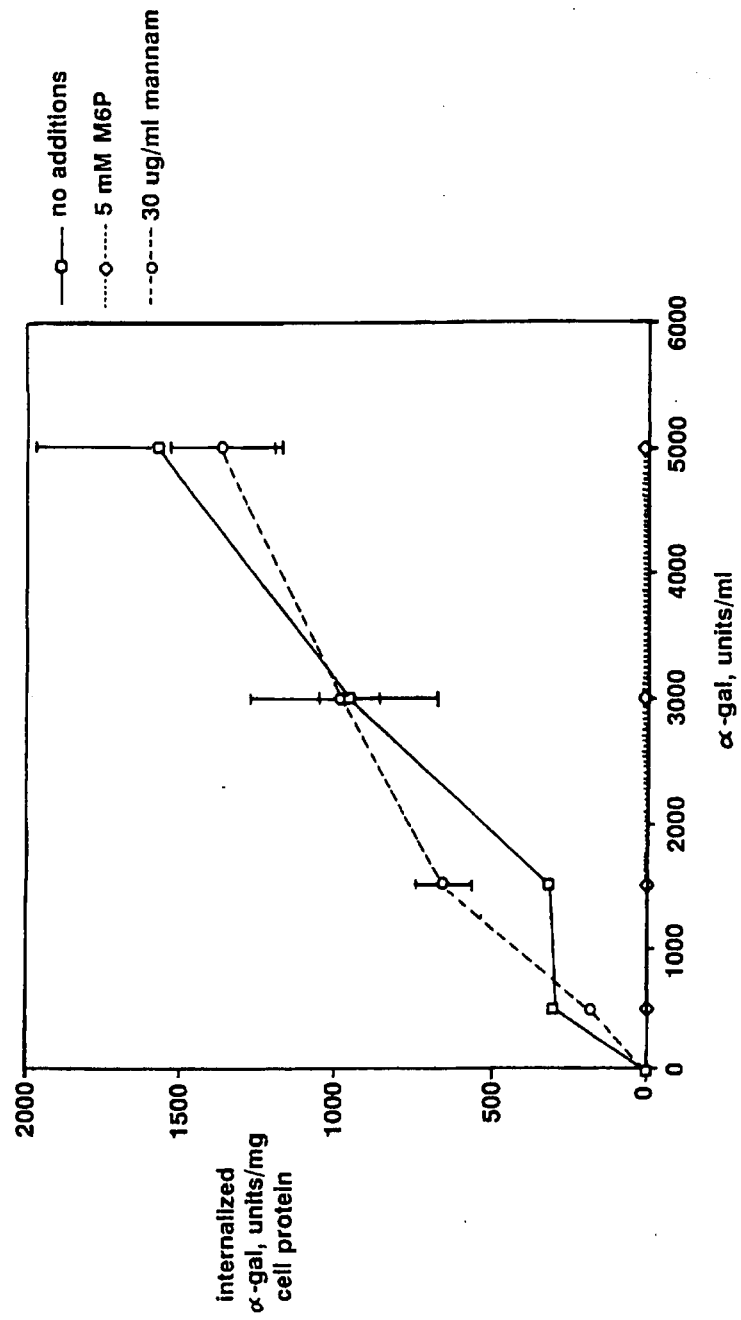
FIG. 4

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FIG. 5

FIG. 6

FIG. 7
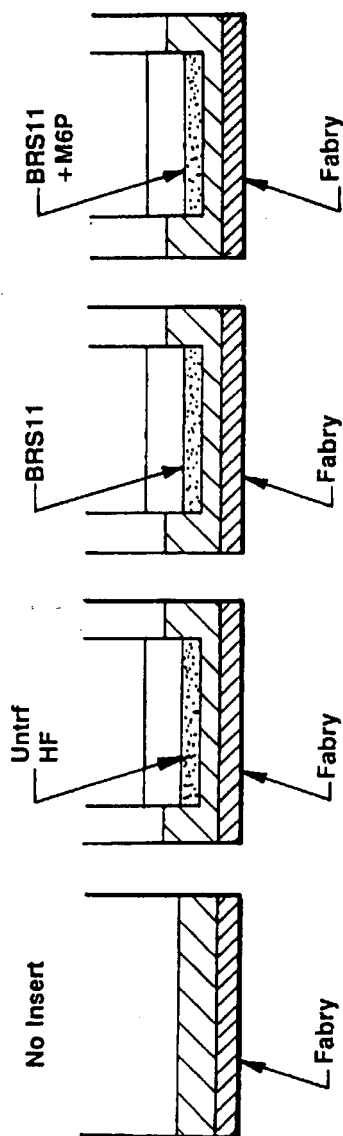
FIG. 8

```
Leu  Asp  Asn  Gly  Leu  Ala  Arg  Thr  Pro  Thr  Met  Gly
Trp  Leu  His  Trp  Glu  Arg  Phe  Met  Cys  Asn  Leu  Asp
Cys  Gln  Glu  Glu  Pro  Asp  Ser  Cys  Ile  Ser  Glu  Lys
Leu  Phe  Met  Glu  Met  Ala  Glu  Leu  Met  Val  Ser  Glu
Gly  Trp  Lys  Asp  Ala  Gly  Tyr  Glu  Tyr  Leu  Cys  Ile
Asp  Asp  Cys  Trp  Met  Ala  Pro  Gln  Arg  Asp  Ser  Glu
Gly  Arg  Leu  Gln  Ala  Asp  Pro  Gln  Arg  Phe  Pro  His
Gly  Ile  Arg  Gln  Leu  Ala  Asn  Tyr  Val  His  Ser  Lys
Gly  Leu  Lys  Leu  Gly  Ile  Tyr  Ala  Asp  Val  Gly  Asn
Lys  Thr  Cys  Ala  Gly  Phe  Pro  Gly  Ser  Phe  Gly  Tyr
Tyr  Asp  Ile  Asp  Ala  Gln  Thr  Phe  Ala  Asp  Trp  Gly
Val  Asp  Leu  Leu  Lys  Phe  Asp  Gly  Cys  Tyr  Cys  Asp
Ser  Leu  Glu  Asn  Leu  Ala  Asp  Gly  Tyr  Lys  His  Met
Ser  Leu  Ala  Leu  Asn  Arg  Thr  Gly  Arg  Ser  Ile  Val
Tyr  Ser  Cys  Glu  Trp  Pro  Leu  Tyr  Met  Trp  Pro  Phe
Gln  Lys  Pro  Asn  Tyr  Thr  Glu  Ile  Arg  Gln  Tyr  Cys
Asn  His  Trp  Arg  Asn  Phe  Ala  Asp  Ile  Asp  Asp  Ser
Trp  Lys  Ser  Ile  Lys  Ser  Ile  Leu  Asp  Trp  Thr  Ser
Phe  Asn  Gln  Glu  Arg  Ile  Val  Asp  Val  Ala  Gly  Pro
Gly  Gly  Trp  Asn  Asp  Pro  Asp  Met  Leu  Val  Ile  Gly
Asn  Phe  Gly  Leu  Ser  Trp  Asn  Gln  Gln  Val  Thr  Gln
Met  Ala  Leu  Trp  Ala  Ile  Met  Ala  Ala  Pro  Leu  Phe
Met  Ser  Asn  Asp  Leu  Arg  His  Ile  Ser  Pro  Gln  Ala
Lys  Ala  Leu  Leu  Gln  Asp  Lys  Asp  Val  Ile  Ala  Ile
Asn  Gln  Asp  Pro  Leu  Gly  Lys  Gln  Gly  Tyr  Gln  Leu
Arg  Gln  Gly  Asp  Asn  Phe  Glu  Val  Trp  Glu  Arg  Pro
Leu  Ser  Gly  Leu  Ala  Trp  Ala  Val  Ala  Met  Ile  Asn
Arg  Gln  Glu  Ile  Gly  Gly  Pro  Arg  Ser  Tyr  Thr  Ile
Ala  Val  Ala  Ser  Leu  Gly  Lys  Gly  Val  Ala  Cys  Asn
Pro  Ala  Cys  Phe  Ile  Thr  Gln  Leu  Leu  Pro  Val  Lys
Arg  Lys  Leu  Gly  Phe  Tyr  Glu  Trp  Thr  Ser  Arg  Leu
Arg  Ser  His  Ile  Asn  Pro  Thr  Gly  Thr  Val  Leu  Leu
Gln  Leu  Glu  Asn  Thr  Met  Gln  Met  Ser  Leu  Lys  Asp
Leu  Leu
```

FIG. 9

ATGGCTACAG <u>GTAAGCGCCC</u> <u>CTAAAATCCC</u> <u>TTTGGGCACA</u>

<u>ATGTGTCCTG</u> <u>AGGGGAGAGG</u> <u>CAGCGACCTG</u> <u>TAGATGGGAC</u>

<u>GGGGGCACTA</u> <u>ACCCTCAGGT</u> <u>TTGGGGCTTC</u> <u>TGAATGTGAG</u>

<u>TATCGCCATG</u> <u>TAAGCCCAGT</u> <u>ATTTGGCCAA</u> <u>TCTCAGAAAG</u>

<u>CTCCTGGTCC</u> <u>CTGGAGGGAT</u> <u>GGAGAGAGAA</u> <u>AAACAAACAG</u>

<u>CTCCTGGAGC</u> <u>AGGGAGAGTG</u> <u>CTGGCCTCTT</u> <u>GCTCTCCGGC</u>

<u>TCCCTCTGTT</u> <u>GCCCTCTGGT</u> <u>TTCTCCCCAG</u> GCTCCCGGAC

GTCCCTGCTC CTGGCTTTTG GCCTGCTCTG CCTGCCCTGG

CTTCAAGAGG GCAGTGCC

FIG. 10

ATGGCTACAG GCTCCCGGAC GTCCCTGCTC CTGGCTTTTG

GCCTGCTCTG CCTGCCCTGG CTTCAAGAGG GCAGTGCC

FIG. 11

Met Ala Thr Gly Ser Arg Thr Ser Leu Leu Leu Ala
Phe Gly Leu Leu Cys Leu Pro Trp Leu Gln Glu Gly
Ser Ala

FIG. 12

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   1 CTGGACAATG GATTGGCAAG GACGCCTACC ATGGGCTGGC TGCACTGGGA GCGCTTCATG

  61 TGCAACCTTG ACTGCCAGGA AGAGGCTGG AGAGGCTGG GTTGAGAAGCT CTTCATGGAG

 121 ATGGCAGAGC TCATGGTCTC AGAAGGCTGG AAGGATGCAG GTTATGAGTA CCTCTGCATT

 181 GATGACTGTT GGATGGCTCC CCAAAGAGAT TCAGAAGGCA GACTTCAGGC AGACCCTCAG

 241 CGCTTTCCTC ATGGGATTCG CCAGCTAGCT AATTATGTTC ACAGCAAAGG ACTGAAGCTA

 301 GGGATTTATG CAGATGTTGG AAATAAAACC TGCGCAGGCT TCCCTGGGAG TTTTGGATAC

 361 TACGACATTG ATGCCCAGAC CTTTGCTGAC TGGGGAGTAG ATCTGCTAAA ATTTGATGGT

 421 TGTTACTGTG ACAGTTTGGA AAATTTGGCA GATGGTTATA AGCACATGTC CTTGGCCCTG

 481 AATAGGACTG GCAGAAGCAT TGTGTACTCC TGTGAGTGGC CTCTTTATAT GTGGCCCTTT

 541 CAAAAGCCCA ATTATACAGA AATCCGACAG TACTGCAATC ACTGGCGAAA TTTTGCTGAC

 601 ATTGATGATT CCTGGAAAAG TATAAAGAGT ATCTTGGACT GGACATCTTT TAACCAGGAG

 661 AGAATTGTTG ATGTTGCTGG ACCAGGGGGT TGGAATGACC CAGATATGTT AGTGATTGGC

 721 AACTTTGGCC TCAGCTGGAA TCAGCAAGTA ACTCAGATGG CCCTCTGGGC TATCATGGCT

 781 GCTCCTTTAT TCATGTCTAA TGACCTCCGA CACATCAGCC CTCAAGCCAA AGCTCTCTT

 841 CAGGATAAGG ACGTAATTGC CATCAATCAG GACCCCTTGG GCAAGCAAGG GTACCAGCTT

 901 AGACAGGGAG ACAACTTTGA AGTGTGGGAA CGACCTCTCT CAGGCTTAGC CTGGGCTGTA

 961 GCTATGATAA ACCGGCAGGA GATTGGTGGA CCTCGGCTCTT ATACCATCGC AGTTGCTTCC

1021 CTGGGTAAAG GAGTCGGCCTG TAATCCTGCC TGCTTCATCA CACAGCTCCT CCCTGTGAAA

1081 AGGAAGGTAG GGTTCTATGA ATGGACTTCA AGGTTAAGAA GTCACATAAA TCCCACAGGC

1141 ACTGTTTTGC TTCAGCTAGA AAATACAATG CAGATGTCAT TAAAGAGCTT ACTTTAA
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FIG. 13